2015-1570

United States Court of Appeals for the Federal Circuit

RAPID LITIGATION MANAGEMENT LTD., FORMERLY CELSIS HOLDINGS, INC., AND IN VITRO, INC.,

Plaintiffs-Appellants,

v.

CELLZDIRECT, INC., A DELAWARE CORPORATION AND WHOLLY-OWNED SUBSIDIARY, AND INVITROGEN CORPORATION, A DELAWARE CORPORATION,

Defendants-Appellees,

Appeal from the United States District Court for the Northern District of Illinois in case no. 10-cv-4053, District Judge Milton I. Shadur

CORRECTED APPELLANTS' BRIEF

Andrew J. Pincus
Paul W. Hughes
MAYER BROWN LLP
1999 K Street NW
Washington, DC 20006

Adam G. Kelly
John A. Cotiguala
LOEB & LOEB LLP
321 North Clark Street, Suite 2300
Chicago, Illinois 60654

Laura A. Wytsma
LOEB & LOEB LLP
10100 Santa Monica Boulevard
Los Angeles, California 90067

Counsel for Plaintiffs-Appellants

CERTIFICATE OF INTEREST

Counsel for Plaintiffs-Appellants Rapid Litigation Management Ltd. and In Vitro, Inc. certify under Federal Circuit Rules 28(a)(1) and 47.4(a) as follows:

1. The full name of every party represented by me is:

   Rapid Litigation Management Ltd. and In Vitro, Inc.

2. The name of the real party in interest (if the party named in the caption is not the real party in interest) represented by me is:

   Rapid Litigation Management Ltd. and In Vitro, Inc. are the real parties in interest.

3. All parent corporations and any publicly held companies that own 10 percent or more of the stock of the party or amicus curiae represented by me are:

   In Vitro, Inc. is a wholly-owned subsidiary of IVT Holdings, Inc.

   Rapid Litigation Management Ltd. has no parent corporation. The only publicly held company that owns 10% or more of Rapid Litigation Management Ltd.'s stock is North Atlantic Smaller Companies Investment Trust PLC.

4. All law firms and the partners or associates that appeared for the party or amicus now represented by me in the trial court or agency or are expected to appear in this court are:

   Loeb & Loeb LLP: Adam G. Kelly, Laura A. Wytsma, John A. Cotiguala
   Mayer Brown LLP: Paul W. Hughes, Andrew J. Pincus
   Dunlap & Codding PC: Jordan A. Sigale, Julie L. Langdon

August 26, 2015

/s/ Adam G. Kelly
Adam G. Kelly
Counsel for Plaintiffs-Appellants

TABLE OF CONTENTS

Page

TABLE OF CONTENTS CONTINUED

Page

TABLE OF AUTHORITIES

Page(s)

Cases

Statutes

Other Authorities

STATEMENT OF RELATED CASES

Two other appeals involving this action were previously before this Court. The first appeal (No. 2010-1547) was decided on January 9, 2012 in *Celsis In Vitro, Inc. v. CellzDirect, Inc.*, 664 F.3d 922 (Fed. Cir. 2012), and the second appeal (No. 2011-1337) was decided on October 21, 2011, in *Celsis In Vitro, Inc. v. CellzDirect, Inc.*, No. 2011-1337 (Fed. Cir. Oct. 21, 2011). There are no pending cases known to counsel that would directly affect or be directly affected by this Court's decision in the present appeal.

JURISDICTIONAL STATEMENT

The district court had jurisdiction over this case under 28 U.S.C. §§ 1331, 1338, and 2201. On March 13, 2005, the district court issued its memorandum opinion and order, granting defendants' motion for summary judgment of invalidity under 35 U.S.C. § 101, and later issued a supplemental opinion on March 16, 2015. IVT timely filed its notice of appeal on April 14, 2015. Therefore, this Court has jurisdiction over this appeal under 28 U.S.C. § 1291.

STATEMENT OF THE ISSUES

Whether the district court erred in determining on summary judgment that the method claims in U.S. Patent No. 7,604,929 are directed to patent ineligible subject matter under Section 101 of 35 United States Code, even though the claims employ a non-naturally occurring cryopreservation process, collectively unknown in the art, to create a non-naturally occurring multi-cryopreserved hepatocyte product.

STATEMENT OF CASE AND FACTS

A. The Background of Hepatocyte Cryopreservation

Scientists continue to advance modern medicine by developing new lifesaving medical procedures and drugs. To bring these procedures and drugs to the public, extensive testing is required to ensure effectiveness and safety. Testing of new drugs focuses on the impact of the substance on the human liver, because every drug taken into the body will eventually pass through the liver during the body's detoxification process. A24 at col. 1:17-29.

Liver cells called hepatocytes contain a number of attributes that make them particularly attractive for use in laboratory studies measuring the effect of new drugs on liver function. A24 at col. 1:16-2:21. In addition, hepatocytes have the potential to preserve liver functions for individuals suffering from liver disease or

liver failure—either through the production of bio-artificial livers or hepatocyte transplantation. A24 at col. 1:59-2:21.

Two problems have significantly limited the use of hepatocytes for these beneficial purposes. First, the supply of hepatocytes is erratic and limited. A24 at col. 2:22-30. Fresh hepatocytes can only be obtained from liver resections or non-transplantable livers of multi-organ donors. A24 at col. 2:25-27. This complete reliance on donations of live livers or liver tissue means that fresh hepatocyte availability is unpredictable. A24 at col. 2:22-35. In addition, the lifespan of hepatocytes is short, which limits their use to the geographic region close to the liver donor. A24 at col. 2:30-35. For both these reasons, research using hepatocytes is wholly dependent on local liver donations—and is forced to start and stop with little advance warning. This unpredictability significantly hinders laboratory studies, which usually require a consistent source of supplies. *See Celsis In Vitro, Inc. v. CellzDirect, Inc..*, 664 F.3d 922, 925 (Fed. Cir. 2012).

To address this problem, and assure a more consistent supply of hepatocytes for these important scientific purposes, scientists have sought to invent techniques for long-term storage of hepatocytes in the laboratory. A24 at col. 2:36-39. The option of cryopreservation (freezing) was unsatisfactory because freezing extensively damages hepatocyte cells. A25 at col. 3:5-32. Because hepatocytes are extremely fragile, once damaged, they often do not recover. A25 at col. 3:5-32.

Cryopreservation may include liquid nitrogen (- 196°C) or frozen nitrogen gas (-150°C). A24 at col. 54-56. Thus, even a single instance of cryopreservation can jeopardize hepatocyte viability. A25 at col. 3:5-32. For this reason, experts in this field met initial attempts to cryopreserve hepatocytes with justifiable skepticism. *Id.*; A2355-56.

The second problem with the use of both fresh and cryopreserved hepatocytes is the biological variation in individual donors. A25 at col. 3:33-46. The results of a test using one or two donors' hepatocytes may not represent the impact of the drug on a larger population. A25 at col. 3:33-46. One solution is to pool hepatocyte samples from a group of donors, which creates a composite hepatocyte sample that is representative of the larger population. A25 at col. 3:46-52.

Yet, as noted above, liver donations are unpredictable, and it is unlikely that a group of liver donors comprising an appropriate pool will occur at the same time. A25 at col. 3:49-52. For example, even if two human livers became available within a single day, there likely would not be enough time to pool the hepatocytes of both prior to cryopreservation. A7991-92 at ¶ 9. As a result, companies were limited to cryopreserving single-donor hepatocytes as they became available. A25 at col. 3:52-56. Researchers were unable to purchase a cryopreserved pool of multi-donor hepatocytes, and instead had to purchase cryopreserved hepatocytes of

single donors until they accumulated enough for an appropriate pool. A25 at col. 3:52-56. But because cryopreserved hepatocytes must be used immediately after thawing, any unused cells were discarded. A7991 at ¶ 8.

B. IVT Develops the Patented LiverPool™ Process

Since beginning as a University of Maryland-sponsored incubator in 1990, IVT has emerged as a world innovator in the field of cryopreserved hepatocyte products.[1] A1232 at ll. 19-25; A8421 ¶ 4. IVT created a novel process to produce its flagship LiverPool™ multi-cryopreserved hepatocyte products. A8421-22 ¶ 6. LiverPool™ provides significant advantages over the prior techniques of purchasing multiple vials of single-donor hepatocytes thawing them and then pooling them together immediately before drug testing. A7993 ¶¶ 13-14. These advantages include time-savings, fewer wasted cells, and performance consistency.

[1] Originally, Celsis In Vitro, Inc. ("Celsis In Vitro") was the assignee of all right, title, and interest in and to the '929 patent. A7664 ¶ 11. But IVT Holdings, Inc. ("IVT Holdings") purchased all of the shares of outstanding capital stock of Celsis In Vitro, and as a result, IVT Holdings' wholly-owned subsidiary, In Vitro, Inc., became the owner of the '929 patent. A8683-84. Under that transaction, Celsis Holdings, Inc., the parent company of Celsis In Vitro, retained an interest in at least past infringement damages to the '929 patent. *Id*. Subsequently, Celsis Holdings, Inc. was joined as a necessary party. A8683-84; A66 at Dkt. 298. Later, Celsis Holdings, Inc. transferred its rights and interests to Rapid Litigation Management Ltd, A8717-18, which was substituted for Celsis Holdings, Inc. A8720. Thus, the plaintiffs are now In Vitro, Inc. and Rapid Litigation Management Ltd. (collectively, "IVT").

A7993 ¶ ¶13-14. In October 2009 IVT received a patent on the process that creates LiverPool™, U.S. Patent No. 7,604,929 ("the '929 patent"). A22-33.

C. The '929 patent

1. Asserted claims 1-9, and 11

Claim 1 recites:

> A method of producing a desired preparation of multi-cryopreserved hepatocytes, said hepatocytes, being capable of being frozen and thawed at least two times, and in which greater than 70% of the hepatocytes of said preparation are viable after the final thaw, said method comprising:
>
> > (A) *subjecting* hepatocytes that have been frozen and thawed to density gradient fractionation to separate viable hepatocytes from non-viable hepatocytes,
> >
> > (B) *recovering* the separated viable hepatocytes, and
> >
> > (C) *cryopreserving* the recovered viable hepatocytes to thereby form said desired preparation of hepatocytes without requiring a density gradient step after thawing the hepatocytes for the second time, wherein the hepatocytes are not plated between the first and second cryopreservations, and wherein greater than 70% of the hepatocytes of said preparation are viable after the final thaw.

A33 at col. 19:56-20:19 (emphasis added). In other words, claim 1 recites three key steps: subjecting thawed hepatocytes to density gradient fractionation, recovering the separated hepatocytes, and cryopreserving for a second time the recovered viable hepatocytes. Dependent claims to claim 1 are directed to the type of density gradient fractionation (claim 2), various hepatocytes (Claims 3 and 4), and viability (Claim 9).

Claim 5 is directed to the methods of claim 1, "wherein said preparation comprises a pooled preparation of hepatocytes of multiple sources." A33 at col. 20:31-33. Inherent in claim 5 is a pooling step. Claim 5 has several dependent claims. A33 at col. 20:34-47, 20:61-62. The multiple sources in claim 5 can be of the same (claim 6) or different (claim 11) gender, race, or health state. A33 at col. 20:34-35, 20:61-62. According to claim 7, "the hepatocytes of said pooled preparation of hepatocytes provide said pooled preparation with a desired level of a metabolic activity." A33 at col. 20:36-38. And under claim 8, "wherein said metabolic activity is selected from the group consisting" of specified enzymatic activities. A33 at col. 20:39-47.

2. The Written Description

The specification teaches that at the time of the invention in 2005, hepatocyte cryopreservation techniques faced serious problems. A25 at col. 3:5-29. One problem was that hepatocyte cryopreservation was widely known to significantly decrease cellular viability. A25 at col. 3:5-29. The ability to recover viable thawed cells would often depend upon numerous factors, including the rate of freezing, the concentration of hepatocytes, the type of cryoprotectant employed, and the final cooling temperature. A24 at col. 2:57-60. And poor recovery techniques of cryopreserved hepatocytes limited the use of such hepatocytes in *in vitro* liver models. A25 at col. 3:30-31.

The specification also teaches that these *in vitro* models faced problems because of the observed variation of liver enzyme expression in hepatocytes from different donors. A25 at col. 3:33-35. And, although pooling samples was one possible solution, limited availability of donor livers prevented usage of hepatocyte cryopreservation techniques to create pooled hepatocyte products. A25 at col. 3:46-53.

3. The Prosecution History

During prosecution the patent examiner expressly addressed the difficulties with hepatocyte cryopreservation:

> The prior art teaches that hepatocytes are extremely difficult to work with and that it is extremely difficult to maintain a sufficient level of viability of hepatocytes during even one round of cryopreservation and thawing. The prior art only discloses methods having one freeze-thaw cycle of hepatocytes, wherein, upon thawing, a gradient centrifugation step is required to remove the non-viable cells … Given the state of the art at the time the invention was made, the instant method would not have been obvious to one of skill in the art.

A2513-14.[2]

[2] A person of ordinary skill in the art of the cryopreservation of hepatocytes "is a person who holds at least a bachelor of science in chemistry, biochemistry, cellular biology, or the like and has one to three years of work experience in the field with primary human hepatocytes. In addition, a person of ordinary skill in the art would consult his colleagues having expertise in the area of cryopreservation of hepatocytes and/or read the published literature concerning the same, should that person find himself over his head." A761 ¶ 26.

D. Prior Proceedings

1. Initial District Court and Federal Circuit Proceedings

In June 2010, IVT filed this action for patent infringement and sought a preliminary injunction, A7662-76; A7706, which was entered that September. A8681-82. This Court affirmed this issuance of that injunction. *Celsis in Vitro*, 664 F.3d at 932. Regarding defendants' invalidity challenge, this Court found that "the present invention is in an art well-known for its unpredictability" and that the "art was a crowded field for many years and yet there was not one reference to multi-cryopreservation." *Celsis*, 664 F.3d at 928 (emphasis in original). This Court also recognized that the prior art teaches away from the claims of the '929 patent: "Moreover, the record shows that the prior art taught away from multiple [cryopreservations]. A single round of [cryopreservation] severely damages hepatocyte cells and results in lower cell viability." *Id*.

2. The PTO Confirms Validity in Post-Grant Review Proceedings

Meanwhile, the PTO instituted an ex parte reexamination of the '929 patent. A4549-61. By early 2012, all asserted claims from the '929 patent were confirmed valid and the PTO again concluded that the prior art taught away from the claimed subject matter:

> The prior art evidences cellular damage produced by cryo-preservation, and a lack of any experimentation with multiply cryopreserved cells. The cumulative prior art fails to establish a prima

> facie case as there is no expectation of success for the method of claim 1. Exhibits A-N and particularly, the Li, Gupta and Strom statements support such conclusions.

A7157. Further supporting its conclusion, the PTO specifically referenced statements from all of the experts, including IVT's Dr. Stephen Strom, who opined that artisans at the time of the '929 invention expected greater loss of cells with every additional cryopreservation. A7157; A5973-74 ¶ 17.

E. The District Court Grants Summary Judgment of Invalidity Under Section 101

In October 2014, LTC moved for summary judgment of invalidity for each asserted claim as lacking patentable subject matter and sufficient written description. A110-11, A115. IVT opposed those arguments. A182-89.

On March 13, 2015, the district court held that the '929 patent claimed patent ineligible subject matter under Section 101, and thus was invalid.[3] A17. As to the first *Mayo* step, the court declined to examine whether claims were directed to a natural phenomenon, A13 at n. 6, but concluded that the '929 patent claims were directed to "an ineligible law of nature: the discovery that hepatocytes are capable of surviving multiple freeze-thaw cycles." A13. The court rested that conclusion on the preamble language, not the body of the claim. *Id.*

[3] The court declined to grant summary judgment of invalidity under Section 112. A16-17.

As to the second *Mayo* step, the court found that "the patented process lacks the requisite inventive concept." A13. The court considered the patent to be "a straightforward application of the truth that hepatocytes are inherently capable of surviving multiple freeze-thaw cycles." A14.

Finally, although recognizing that the patent claims were more narrowly drawn than the patents-at-issue in *Mayo* and *Alice* because they do "not lock up the natural law in its entirety," the court nevertheless found that the claims were sufficiently preemptive. A15-16. The court acknowledged that other methods existed for creating multi-cryopreserved hepatocyte preparations (*e.g.*, elutriation centrifugation), but reasoned that if the patent law permitted "a lock on a narrow albeit routine combination of steps, different combinations of other routine steps would also be patent-eligible." A15-16. (internal quotations omitted). "Put another way, if one were allowed to own a slice of the preemptive pie, that would pave the way for multiple others to claim the rest of that pie." A16.

Subsequently, the district court issued a supplemental opinion to "provide a more brief summary of its fundamental holding" while the parties resolved confidentiality redaction issues. A18-19. The court again identified the "natural fact that, in a normal population of hepatocytes … some sub-population is capable of surviving the process of being frozen and thawed at least two times." A19. "The remaining claim elements consist of the application of only well-understood,

routine, and conventional cell separation and cryopreservation steps admittedly in common use long before the time of the claimed inventions." *Id*. For this reason, the court concluded that the claims are nothing more than the "discovery" that "some hepatocytes can be frozen multiple times." *Id*.

SUMMARY OF THE ARGUMENT

In the early 1990s, a few scientists began experimenting with cryopreservation techniques for hepatocyte cells in order to use them as testing models for drug research. At first, the scientific community was slow to adopt these models, due to the severe fragility of hepatocytes and the potential for skewed test results. But eventually, scientists began using such assays, though these problems persisted.

Then, in 2005, scientists at IVT created a new process for creating multi-cryopreserved hepatocyte products using hepatocytes from different donors. This process allowed IVT to create 5- and 10-donor hepatocyte pools called LiverPool™--an advance that led IVT to become the worldwide leader in the hepatocyte field. That would not have been possible without the asserted claims that are the subject of this appeal, and the exclusivity they once afforded.

These claims recite a novel method performed by lab technicians to create a non-naturally occurring product—a multi-cryopreserved hepatocyte product, which is typically pooled from multiple sources, having at least 70% viability. Despite

having previously found that IVT's patent was sufficiently valid to support a preliminary injunction, affirmed by this Court in *Celsis In Vitro, Inc. v. CellzDirect, Inc.*, 664 F.3d 922 (Fed. Cir. 2012), the district court subsequently concluded that none of the claims were patent eligible under 35 U.S.C. § 101.

The question here is straightforward—may IVT enforce its patent to enjoy its remaining exclusivity or does Section 101 effectively say that IVT cannot patent a process which was not taught anywhere in the prior art and defied conventional knowledge in the cryopreserved hepatocyte field in 2005?

This Court should resolve this question in favor of IVT. Indeed, the district court's opinion dramatically expands the judicially-created "natural law exception" under Section 101 in a manner that would deny patent eligibility to a wide swath of pharmaceutical inventions, chemical inventions, and any other innovation produced by applying to a naturally-occurring substance a process to which it is not subjected in nature in order to produce a non-naturally occurring product.

Under the first *Mayo/Alice* step, the district court failed to appreciate that the claims are directed to a non-naturally occurring manufacturing method. The claims begin with non-naturally occurring materials (*i.e.*, cryopreserved hepatocytes) and end with different non-naturally occurring products (*i.e.*, pooled, multi-cryopreserved hepatocytes). A person, not nature, creates these products by

using processes to which the materials are not subjected in nature. That is the essence of invention.

The claims also satisfy the second *Mayo/Alice* step by sufficiently reciting an inventive concept—an unconventional combination of steps to create a non-naturally occurring product. The prior art and the patent teach that the combination of the claimed steps was unknown, let alone well-known. Even though individual elements were known, it is the non-conventional combination that is patentable under *Diamond v. Diehr*.

Yet the district court's misunderstanding of what was conventional and well-known at the time rested on its decision to ignore the patent specification and the art, instead relying on contrary co-inventor testimony. That was clear error.

And despite conceding that the claims did not preempt others from making multi-cryopreserved hepatocytes, the district court misapplied preemption principles under *In re BRAC-1 and BRAC-2 Litigation* to find that the claims were somehow sufficiently preemptive. But that preemption logic is unsupportable given the clear evidence in the record of other known methods for making multi-cryopreserved hepatocytes—one of which was recognized by this Court in *Celsis In Vitro, Inc. v. CellzDirect, Inc.,* no. 2011-1337 (Fed. Cir. Oct. 21, 2011). As this Court already found, there are alternative, non-infringing ways to create multi cryopreserved hepatocytes.

Accordingly, because the claims are patentable under Section 101, this Court should reverse the district court's decision and remand for further proceedings.

ARGUMENT

I. STANDARD OF REVIEW

This Court reviews the grant of summary judgment under regional circuit law, which here is the Seventh Circuit. *Teva Pharm. Indus. Ltd. v. Astrazeneca Pharms. LP*, 661 F.3d 1378, 1381 (Fed. Cir. 2011). Under Seventh Circuit law, summary judgment rulings are reviewed de novo. *See Hanover Ins. Co. v. Northern Bldg. Co.*, 751 F.3d 788, 791 (7th Cir. 2014). In addition this Court reviews de novo patent eligibility decisions under Section 101. *In re BRCA1- and BRCA2-Based Hereditary Cancer Test Patent Litig.*, 774 F.3d 755, 759 (Fed. Cir. 2014). Under Section 282, patents are presumed valid, absent clear and convincing evidence to the contrary. *See, e.g., CLS Bank Int'l v. Alice Corp. Pty. Ltd.*, 717 F.3d 1269, 1284, 1304-05 (Fed. Cir. 2013).

II. THE DISTRICT COURT ERRED IN GRANTING SUMMARY JUDGMENT OF INVALIDITY UNDER SECTION 101

A. The *Mayo*/*Alice* Framework

Section 101 of the Patent Act defines patent eligible subject matter:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

35 U.S.C. § 101. These categories of patent eligible subject matter have always been interpreted broadly to reflect congressional intent that "the patent laws would be given a wide scope." *Bilski v. Kappos*, 561 U.S. 593, 601 (2010).

But Section 101 is subject to three specific exceptions: laws of nature, physical phenomena, and abstract ideas, which are not "patent-eligibl[e] principles." *Bilski*, 561 U.S. at 601. They "are the basic tools of scientific and technological work" and thus "the grant of a patent might tend to impede innovation more than it would tend to promote it." *Mayo Collaborative Servs. v. Prometheus Labs., Inc.*, 132 S. Ct. 1289, 1293 (2012).

These exceptions are necessarily narrow: "too broad an interpretation of this exclusionary principle could eviscerate patent law," because "'all inventions at some level embody, use, reflect, rest upon, or apply laws of nature, natural phenomena, or abstract ideas.'" *Mayo*, 132 S. Ct. at 1293. Accordingly, courts must "tread carefully in construing this exclusionary principle lest it swallow all of patent law." *Alice Corp. Pty. Ltd. v. CLS Bank Int'l*, 134 S. Ct. 2347, 2354 (2014).

In *Mayo* and *Alice*, the Supreme Court articulated a two-step framework for distinguishing between patent eligible and patent ineligible claims. *First*, a court must "determine whether the claims at issue are directed to a patent-ineligible concept." *Alice*, 134 S. Ct. at 2355. If they are not so directed, then the claims satisfy Section 101's patentability requirement without any further analysis.

Second, if the patent claims are directed to a patent ineligible concept, then a court must next consider the elements of each claim both individually and "as an ordered combination" to determine whether elements other than the patent ineligible concept "transform the nature of the claim" into a patent eligible application. *Mayo*, 132 S. Ct. at 1297-8. *Mayo* described this second step as a search for an "inventive concept"—*i.e.*, an element or combination of elements that is "sufficient to ensure that the patent in practice amounts to significantly more than a patent upon the [ineligible concept] itself." *Id*. at 1294.

B. *Mayo*/*Alice* Step 1: The Claims Recite A Method For Creating A Non-Naturally Occurring Composition Or Manufacture, Not A Patent Ineligible Law Of Nature.

The district court "conclude[d] that the patent is directed to an ineligible law of nature: the discovery that hepatocytes are capable of surviving multiple freeze-thaw cycles." A13. Although the district court cited the PTO guidelines for support that it must analyze what subject matter is recited in the claims, it solely relied on the preamble. A13. In other words, the district court ignored the words that legally define the invention. *Phillips v. AWH Corp.*, 415 F.3d 1303, 1312 (Fed. Cir. 2005). Therefore the court's analysis was flawed from the start.

But the court failed to recognize (*see* A13), entirely, that the claims are patent eligible under the first step of *Mayo*/*Alice* for a separate reason: the claims are directed to a "'nonnaturally occurring manufacture or composition of matter.'" *Ass'n for Molecular Pathology v. Myriad Genetics Inc.*, 133 S. Ct. at 2107, 2117 (2013) (quoting *Diamond v. Chakrabarty*, 447 U.S. 303, 309-10 (1980)).

Of course, the "discovery" of a naturally-occurring phenomenon by itself is not patent eligible. *Myriad*, 133 S. Ct. at 2117. The Supreme Court held in *Funk Brothers* that "a mixture of naturally occurring strains of bacteria that helped leguminous plants take nitrogen from the air and fix it in the soil" "was not patent eligible because the patent holder did not alter the bacteria in any way." *Myriad*, 133 S. Ct. at 2117 (discussing *Funk Brothers Seed Co. v. Kalo Inoculant Co.*, 333

U.S. 127 (1948)). *Funk Brothers* concluded that combinations of bacteria "serve the ends nature originally provided and *act quite independently of any effort of the patentee*." *Funk Brothers*, 333 U.S. at 131. Accordingly, there was no "invention." *Id*. at 132.

Myriad also highlighted the critical distinction between "discovery" and "invention." The Court considered whether a sequence of DNA is patent eligible. By identifying the location of certain aspects of DNA, "Myriad did not create or alter any of the genetic information encoded in the BRCA1 and BRCA2 genes." *Myriad*, 133 S. Ct. at 2116. Myriad's claim was a discovery, not an invention. Myriad argued that "the location of the gene was unknown until Myriad found it among the approximately eight million nucleotide pairs contained in a subpart of chromosome 17." *Id*. at 2117-18. But, the Court explained, "extensive research efforts" are not alone sufficient "to satisfy the demands of § 101." *Id*. at 2118.

By contrast, the Court found that claims directed to cDNA molecules *were* patent eligible. *Myriad*, 133 S. Ct. at 2119. "[C]reation of a cDNA sequence from mRNA results in an exons-only molecule that is *not naturally occurring*." *Id.* (emphasis added). In finding the invention requirement satisfied, the Court specifically rejected the argument that a cDNA molecule was patent ineligible, because "'[t]he nucleotide sequence of cDNA is dictated by nature, not by the lab technician.'" *Id*. Even though laws of nature controlled the creation of cDNA, the

Court concluded that "the lab technician unquestionably creates something new when cDNA is made." *Id*. Justice Scalia, concurring in part and concurring in the judgment, agreed that cDNA is patent eligible because it "is a synthetic creation not normally present in nature." *Id*. at 2120.

Against this background, the claims of the '929 patent are patent eligible under Section 101. They are methods for subjecting non-natural substances to extreme conditions that do not occur in nature to obtain a product that does not occur in nature. The claimed methods begin with cryopreserved hepatocytes (a product which does not itself exist in nature), and then require a lab technician to create a *twice* cryopreserved hepatocyte preparation by, among other things, (1) thawing the previously-frozen cells, (2) separating the viable thawed cells from the non-viable cells using a density gradient fractionation procedure, (3) often pooling the cells from multiple donors, and (4) then subjecting those cells to cryopreservation in a manner that, following a second thaw, will retain a viability rate of at least 70%. A33 at col. 19:62-20:20. Accordingly, these steps direct the preparation of specialized kind of twice cryopreserved hepatocyte cells. (A lay observer would view these preparations as akin to pink-colored ice in a tube.)

This preparation, which certainly does not exist in nature, has quite unique properties, properties that in turn do not exist in nature. For example, the twice-cryopreserved hepatocyte cell preparation produced by these methods retains its

useful viability for a significantly longer period of time than would a preparation of non-cryopreserved hepatocyte cells. A24 at col. 2:30-60. Because the preparations often involve pooled cells, the preparations are vastly more useful for research purposes because they represent theoretical "average" compositions having metabolic enzyme function approximate to a freshly isolated hepatocytes. A25 at col. 3:33-49; A29 at col. 11:2-27; A7993 at ¶¶ 13-14. And, because of the steps required in preparing this preparation, the twice-cryopreserved hepatocyte preparation may be thawed to result in cells with greater than 70 percent viability—a preferred viability standard. A28 at col. 10:61-64.

For these reasons, the patented preparation of twice-cryopreserved hepatocyte cells does not merely display "qualities [that] are the work of nature unaltered by the hand of man." *In re Roslin Inst. (Edinburgh)*, 750 F.3d 1333, 1336 (Fed. Cir. 2014). Rather, it is a new product displaying properties that would *never* exist, but for the hand of man. There is no naturally occurring hepatocyte product that can be stored for lengthy periods of time and then, once allowed to thaw, will result in a pool of hepatocyte cells from multiple donors with a viability ratio greater than 70%. The product produced by the claimed method is instead the work of human ingenuity that results in a composition or manufacture that "is not naturally occurring," *Myriad*, 133 S. Ct. at 2111, and is thus patent eligible. Like the cDNA in *Myriad*, and unlike the bacteria product in *Funk Brothers*, "the lab

technician unquestionably creates something new," *id*. at 2119—that is, the technician produces "a synthetic creation not normally present in nature." *Id*. at 2120 (Scalia, J., concurring in part, and concurring in judgment).

This transformed group of hepatocyte cells is not excluded from Section 101 because somehow it is, like the preparation in *Funk Brothers*, nothing "more than an advance in … packaging." 333 U.S. at 131. The claims use severe temperature changes (*e.g.*, ambient to - 196°C) and density gradient fractionation to manipulate the hepatocyte cells to produce a new, useful product (*i.e.*, a preparation of at least twice-cryopreserved cells having at least 70 percent viability) that exists nowhere in nature. In this way, the "machine-or-transformation test," which serves as "an important and useful clue," strongly confirms that the claims here are patent eligible. *Bilski v. Kappos*, 561 U.S. 593, 603 (2010).

The district court appeared to conclude that Section 101 rendered the claims patent ineligible because natural laws dictate how naturally-occurring cells will be affected by the non-natural processes applied to those cells. But that broad proposition would render non-patentable virtually any scientific discovery. The history of invention rests entirely on subjecting naturally-occurring substances to processes that they do not occur in nature to produce a new product that does not occur in nature—whether that creation is a new drug, glue, or any other product.

Of course, natural laws dictate how the non-natural processes will affect the substance in question; that is why they are natural "laws." But to hold that Section 101 excludes an inventor's use of processes to create a substance not found in nature would severely and improperly constrict the scope of patentability.

In sum, the district court erred by concluding that the claims are directed to an ineligible natural law. Because the claims qualify as "a nonnaturally occurring manufacture or composition of matter," the claims are directed to patent eligible subject matter under the first step of the *Mayo*/*Alice* inquiry. *Myriad*, 133 S. Ct. at 2117. Therefore, the claims satisfy Section 101 and the inquiry may end here.

C. *Mayo/Alice* Step 2: The Claims, When Properly Viewed as a Whole, Recite an "Inventive Concept" under *Mayo*.

The claims also satisfy the second step of the *Mayo/Alice* framework, because they sufficiently recite an inventive concept—the unconventional combination of steps to create a non-naturally occurring product. This result is confirmed by the fact that, if the claims implicate a law of nature at all, they do not unduly preempt that asserted law of nature.

1. The district court failed to analyze each challenged claim.

The district court mistakenly concluded that neither party addressed the dependent claims. A15. But LTC challenged all claims, not just claim 1. A110-11. And IVT opposed each challenge by clearly addressing, at minimum, the

limitations contained in claims 1 and 5. A187. IVT expressly argued that a combination of steps involving pooling (*i.e.*, claim 5, which depends from claim 1) was certainly patent eligible subject matter. A187. Therefore, the district court erred in limiting its analysis to claim 1, and this Court should consider all claims.

2. The district court erroneously applied an expansive view of "conventional" activity to find a lack of inventive concept.

The second step of the *Mayo*/*Alice* framework requires a court to determine whether a patent claim that is otherwise directed to a patent ineligible concept, such as a law of nature, is rendered patent eligible by virtue of an "inventive concept." *Alice Corp.*, 134 S. Ct. at 2357. In considering whether the patents do "something more" than claim "the building blocks of human ingenuity" (*id*. at 2354 (quotation omitted)), the Supreme Court has reasoned that "[s]imply appending conventional steps, specified at a high level of generality, [is] not enough to supply an inventive concept." *Id*. at 2357. That is, "well-understood, routine, conventional activity already engaged in by the scientific community" cannot supply the inventive concept. *Mayo*, 132 S. Ct. at 1298.

Mayo and *Alice* require that activity must not just be known, but "*well*-known" before characterizing it as "conventional" under the second step of the inquiry. *Mayo*, 132 S. Ct. at 1298 (emphasis added). "[N]either Mayo nor any other precedent defines conventional elements to include ***everything*** found in prior

art." *Cal. Inst. of Tech. v. Hughes Commc'ns Inc.*, 59 F. Supp. 3d 974, 989 (C.D. Cal. 2014) (emphasis added). An element is "well-understood" only when it is routinely practiced in the relevant field or industry—that is, when the element is "conventional." *Mayo*, 132 S. Ct. at 1298. In applying this standard, courts look to the patent specification and expert evidence as to what was "known in the literature" at the time of the invention. *Ariosa Diagnostics, Inc.*, 788 F.3d at 1377.

The high bar for finding activity to be "conventional" rests on the structure of the Patent Act itself. If evidence of an activity or element in the prior art were sufficient, then Section 101 would swallow whole the separate analysis under Sections 102 and 103.

This distinction between "conventional," "well-understood" activity, and anything referenced in the prior art is particularly important in the context of process claims. The Supreme Court has long recognized that "a new combination of steps in a process may be patentable even though all the constituents of the combination were well known and in common use before the combination was made." *Diamond v. Diehr*, 450 U.S. 175, 187 (1981) (finding that although that patent claimed the Arrhenius equation, the process as a whole with additional steps was patentable). The mere fact that each step was arguably recognized in the prior art is not dispositive—the combination of steps must have been "conventional."

Against this proper framework, the district court should have concluded that the second step of *Mayo* and *Alice* is satisfied here for at least two reasons. *First*, the application of a second cryopreservation step was plainly not conventional or well-understood at the time. The district court recognized, but then discounted entirely, that the "prior art taught away from multiple [cryopreservations]." A14. In the court's estimation, once the patentee determined that hepatocyte cells could be frozen multiple times, the patentee "reapplied a well-understood freezing process." *Id*.

But while cryopreservation may have been understood as a general matter, *nothing* in the record demonstrates that it was "well-understood" or "conventional" to apply a subsequent cryopreservation step to hepatocytes that had already been frozen and thawed. Indeed, there is little doubt that the prior art expressly taught away from the claimed method steps as a whole. *See*, *supra*, 8-9.

For example, *Terry* disclosed a retrospective description of the unpredictable hepatocyte cryopreservation art. A2270-80. It taught away from cryopreserving hepatocytes, even one time, by explaining the negative effects cryopreservation had on hepatocyte health. A2274-75. Although *Ostrowska* taught that one could successfully freeze and thaw hepatocytes only one time, A8015, it failed to teach multi-cryopreservation or pooling. And while *Shibata* taught that one could pool thawed hepatocytes, it failed to teach that one could cryopreserve the pool. A8036.

Likewise, the written description supports the conclusion that the second cryopreservation step was not "well-known" in 2005. Back then, hepatocyte cryopreservation techniques faced serious problems. A25 at col. 3:5-29. For example, cryopreservation was widely known to significantly decrease hepatocyte viability. A25 at col. 3:5-29. The ability to recover viable thawed hepatocytes would often depend upon numerous factors, including the rate of freezing, the concentration of hepatocytes, the type of cryoprotectant employed, and the final cooling temperature. A24 at col. 2:57-60. And poor recovery techniques of cryopreserved hepatocytes limited the use of such hepatocytes in *in vitro* liver models. A25 at col. 3:30-32. These *in vitro* models faced problems in providing the variation of liver enzyme expression observed from different donors. A25 at col. 3:33-35. Although pooling fresh samples was one possible solution, limited donor livers prevented usage of hepatocyte cryopreservation techniques to create pooled hepatocyte products. A25 at col. 3:46-53.

Taken together, none of the prior art references, as confirmed by the specification, taught or suggested the use of *a second* cryopreservation step. Rather, they taught away from combining those steps. Those teachings are strong indicia that the addition of the second cryopreservation step is non-conventional activity. This conclusion is congruous with how the patent examiner viewed the claimed methods. A2513-14; A7157.

Moreover, the district court's reliance upon the deposition testimony of co-inventor James Hardy did not support its conclusion. The district court pointed to Mr. Hardy's testimony that hepatocyte cryopreservation was "well understood." A14. But that testimony in a vacuum contradicts the written description, which teaches the problems with hepatocyte cryopreservation. And that testimony contradicts the teachings in the prior art, namely, *Ostrowska*, *Shibata*, and *Terry*. Whether a single round of hepatocyte cryopreservation was known misses the point. The question is whether the combination of the claimed steps was known. *Diehr*, 450 U.S. at 188-189. And the answer is that the claimed combination was certainly *unknown* at the time of the invention; it cut against the grain of scientific thought as disclosed in prior art. A2513-14; A7157; A7754-55 at ¶¶ 106-107.

Second, the district court failed to consider the steps of the claimed method as a whole. Here, the combination of steps demonstrates that the methods recite substantially more than mere "conventional" activity. For example, in claims 1 and 5, the claims require:

> Step 1: "subjecting hepatocytes that have been frozen and thawed to density gradient fractionation to separate viable hepatocytes from non-viable hepatocytes." A33 at col. 19:62-64.
>
> Step 2: "recovering the separated viable hepatocytes." A33 at col. 20:12.
>
> Step 3: *without* plating the cells, "cryopreserving the recovered viable hepatocytes." A33 at col. 20:13-18. The third step must be

> performed in such a way that "greater than 70% of the hepatocytes of said preparation are viable after the final thaw," "*without* requiring a density gradient step after thawing the hepatocytes for the second time." A33 at col. 20:15-20 (emphasis added).
>
> Additional step of dependent claim 5: cells of *multiple* donors are pooled in creating the twice cryopreserved cell preparation. A33 at col. 20:31-33 (emphasis added).

As discussed (*supra*, 27), the district court analyzed solely the second cryopreservation step. Yet, it did not find that *any* of the other steps are "conventional" activity. For example, it did not consider whether it is conventional to conduct a second cryopreservation *without* plating the cells between the first and the second thaws, as these claims require. A33 at col. 20:16-18. And the court did not consider whether it is conventional to require, after a second thaw, that the cells reach a viability of greater than 70 percent *without* requiring an additional density gradient step, as these claims require. A33 at col. 20:15-20.

Simply put, there is no basis in this record to conclude that this combination of steps was conventional or well-understood in the industry. There is no evidence that any prior art contained these steps whatsoever, much less that they had become the sort of industry-standard practice that may be as "well-understood, routine, conventional activity already engaged in by the scientific community." *Mayo*, 132 S. Ct. at 1298. And "when viewed as a whole," it is apparent that these

steps together direct the creation of a new, useful, and previously unknown composition—the preparation of twice-cryopreserved hepatocytes having at least 70% viability—that is the innovation within the protection of the patent law. *Id.*

3. The claims do not broadly preempt all methods for creating hepatocyte preparations—or even all methods for twice-cryopreserved preparations.

The district court's erroneous Section 101 analysis is confirmed by assessing the preemptive effect of the claims at issue. As the Supreme Court has explained, the Section 101 analysis turns on how the claims would "inhibit further discovery." *Alice Corp.*, 134 S. Ct. at 2354 (quotation omitted). One important way to determine whether claims would have this impermissible effect is to analyze whether they "too broadly preempt the use of a natural law." *Mayo*, 132 S. Ct. at 1294. Claims that "do not attempt to preempt every application" of the patent ineligible concept generally satisfy the Section 101 threshold. *DDR Holdings, LLC v. Hotels.com, L.P.*, 773 F.3d 1245, 1259 (Fed. Cir. 2014). As explained in *Ultramercial* in the context of Section 101's exclusion of abstract ideas, "[i]t is not the breadth or narrowness of the abstract idea that is relevant, but whether the claim covers every practical application of that abstract idea." *Ultramercial, Inc. v. Hulu, LLC*, 722 F.3d 1335, 1346 (Fed. Cir. 2013).

Here, the district court candidly acknowledged that the claims at issue are "more narrowly drawn than the patents at issue in *Mayo* and *Alice* because [they

do] not lock up the natural law in its entirety." A15. This conclusion is plainly correct: the claimed methods require several steps that limit them far more narrowly than claiming every application of twice-cryopreserved hepatocyte cells. *First*, the method requires the use of density gradient fractionation, although other methods for separating cells are available. A33 at col. 19:62-64; A8891-95; A8722-23. *Second*, the hepatocyte cells are not plated between the first and the second cryopreservations. A33 at col. 20:16-18. *Third*, the preparation requires achievement of at least a 70 percent cell viability following the second thaw. A33 at col. 20:18-20. And *fourth*, claim 5 limits the process further to involving hepatocyte cells pooled from multiple donors. A33 at col. 20:31-33.

Indeed, as the district court itself recognized, the defendant "has already managed to engineer around the patent by using a different mechanism for sorting viable from nonviable cells called elutriation." A15. Thus, the claims do not prevent others from developing new ways to create multi-cryopreserved hepatocytes in the future—which is the key concern in the preemption analysis. *See Mayo*, 132 S. Ct. at 1302 (finding the claims invalid because they "cover[ed] all processes that make use of the correlations after measuring metabolites, including later developed processes that measure metabolite levels in new ways").

The ineligible claims in *Mayo* provide a contrasting example of impermissible preemption. Those claims not only foreclosed the use of the

correlation in the claimed method, but also foreclosed *any* other way of utilizing the correlation. The claimed "administering" and "determining" steps were necessary prerequisites to any use of the correlation at all—one could not consider the correlation without administering the drug and measuring the metabolites. *Mayo*, 132 S. Ct. at 1298 ("Anyone who wants to make use of these laws must first administer a thiopurine drug and measure the resulting metabolite concentrations, and so the combination amounts to nothing significantly more than an instruction to doctors to apply the applicable laws when treating their patients."). Thus the claim prevented others from using the correlation for any purpose at all, even from further developing it in other ways.

Further, the claims in *Mayo* impermissibly preempted the natural law, because any application of the law necessarily required the recited steps of administering a drug and determining metabolite levels. *Mayo*, 132 S. Ct. at 1299-1300 ("And since they are steps that must be taken in order to apply the laws in question, the effect is simply to tell doctors to apply the law somehow when treating their patients."); *accord Ultramercial*, 722 F.3d at 1348 ("[T]he Supreme Court's reference to 'inventiveness' in *Prometheus* can be read as shorthand for its inquiry into whether implementing the abstract idea in the context of the claimed invention inherently requires the recited steps.").

A similar preemption concern was recently present in *Ariosa Diagnostics, Inc. v. Sequenom, Inc.*, 788 F.3d 1371 (Fed. Cir. 2015). There, the issue was whether the claimed methods were patent eligible applications of a natural phenomenon, specifically a method for detecting paternally-inherited cffDNA. 788 F.3d at 1377. The court found that "[u]sing methods like PCR to amplify and detect cffDNA was well-understood, routine, and conventional activity in 1997." *Id*. The methods amounted "to a general instruction to doctors to apply routine, convention techniques when seeking to detect cffDNA." *Id*. "Because the method steps were well-understood, conventional and routine, the method of detecting paternally inherited cffDNA is not new and useful." *Id*. at 1377. Rather, "[t]he only subject matter new and useful. . .was the discovery of the presence of cffDNA in maternal plasma or serum." *Id*. There, the claims *also* failed the preemption inquiry and were thus claim patent ineligible subject matter. *Id*. at 1379.

Here, by contrast, there exist several methods to practice the alleged law of nature that do not infringe the claims. A8891-95; A8722-23. And the district court acknowledged this fact. A15. Thus the preemption inquiry provides substantial evidence confirming that these method claims are patent eligible.

Finally, the district court cited *In re BRAC-1 and BRAC-2 Litigation* and expressed concern that, "if one were to own a slice of the preemptive pie, that would pave the way for multiple others to claim the rest of that pie." A15-16. For

this reason, the court found that the limited preemptive effect of these claims did not demonstrate their patent eligibility. *Id*.

The district court's observation is just wrong, given the practical reality here. Once claims recite an expressed method for obtaining a result, others are barred under Section 103 from patenting different but obvious methods for obtaining the same result. So, there is little concern that others will "claim the rest of [the] pie."

Moreover, the claim limitations implicated here—*i.e.*, the density gradient fractionation, the lack of plating between the cryopreservation steps, the required 70 percent viability of hepatocyte cells, and (in the dependent claim) the pooling of cells from different donors—would not permit such a "segmenting" of all applications of a natural law. Rather, there are numerous permutations of limits that would avoid the claimed methods. Indeed, on a prior appeal in this case, this Court already identified a particular kind of alternative method for accomplishing the claimed results, using an elutriation method to separate hepatocytes rather than density gradient fractionation, that does not infringe the method claims. *See Celsis In Vitro*, No. 2011-1337 (Fed. Cir. Oct. 21, 2011). This is not a circumstance in which there is a small number of possible options for performing a method, and the patentee has attempted to claim one broad swath.

As a legal matter, moreover, the district court's reasoning contradicts *Diehr*'s holding that a new combination of steps may be patentable even though all

the constituents of the combinations were well known and in common use before the combination was made. *Diehr*, 450 U.S. at 187.

III. CONCLUSION

As this Court previously concluded, the ’929 patent is not invalid. The district court erred in granting summary judgment of invalidity under Section 101. Judgment should be reversed and the case remanded for further proceedings.

August 26, 2015

Respectfully submitted,

/s/ Adam G. Kelly

Adam G. Kelly
John A. Cotiguala
LOEB & LOEB LLP
321 North Clark Street, Suite 2300
Chicago, IL 60654

Laura A. Wytsma
LOEB & LOEB LLP
10100 Santa Monica Boulevard
Los Angeles, California 90067

Andrew J. Pincus
Paul W. Hughes
MAYER BROWN LLP
1999 K Street NW
Washington, DC 20006

Counsel for Plaintiffs-Appellants

CERTIFICATE OF COMPLIANCE

Counsel for Plaintiffs-Appellants certifies that this brief has a proportionally spaced 14-point typeface, and contains 7,759 words, based on the "Word Count" feature of Microsoft WORD 2010, including footnotes and endnotes. Under Fed. R. App. P. 32(a)(7)(B)(iii) and Fed. Cir. R. 32(b), this word count does not include the words contained in the Certificate of Interest, Table of Contents, Table of Authorities, and Abbreviations.

August 26, 2015 By: /s/ Adam G. Kelly
Adam G. Kelly

Adam G. Kelly, Esq.
LOEB & LOEB LLP
321 North Clark Street, Suite 2300
Chicago, IL 60654
Telephone: (312) 464-3100

Attorneys for Plaintiffs-Appellants

CERTIFICATE OF SERVICE

I certify that a true and correct copy of the foregoing **CORRECTED APPELLANTS' BRIEF** on behalf of Plaintiffs-Appellants Rapid Litigation Management and In Vitro, Inc. were served via ECF filing on August 26, 2015 to the following counsel:

David G. Mangum
C. Kevin Speirs
Parsons Behle & Latimer
201 South Main Street, Suite 1800
Salt Lake City, UT 84111
(801) 532-1234

Counsel for Defendants-Appellees

By: /s/ Adam G. Kelly
Adam G. Kelly

Adam G. Kelly, Esq.
LOEB & LOEB LLP
321 North Clark Street, Suite 2300
Chicago, IL 60654
Telephone: (312) 464-3100

Attorneys for Plaintiffs-Appellants

ADDENDUM

TABLE OF CONTENTS

Addendum Pursuant to Federal Circuit Rule 28(a)(12)

IN THE UNITED STATES DISTRICT COURT
FOR THE NORTHERN DISTRICT OF ILLINOIS
EASTERN DIVISION

CELSIS IN VITRO, INC.,	)	
	)	
Plaintiff,	)	
	)	
v.	)	Case No. 10 C 4053
	)	
CELLZDIRECT, INC., a Delaware Corporation and wholly-owned subsidiary of **INVITROGEN CORPORATION**; and **INVITROGEN CORPORATION**, a Delaware Corporation,	)	
	)	
Defendants.	)	

MEMORANDUM OPINION AND ORDER

Defendants CellzDirect, Inc. and Invitrogen Corp. (hereafter collectivized as "LTC," the corporation that has succeeded to their interests) bring a motion for summary judgment of patent invalidity under 35 U.S.C. §§ 101 and 112 (Dkt. 335).[1] Also pending is LTC's Motion To Limit Damages to a Reasonable Royalty on LTC's Accused Sales (Dkt. 337). Because this Court finds the patent at issue invalid under Section 101, LTC's Dkt. 335 motion is granted and its second Dkt. 337 motion is consequently denied as moot.

Standard of Review

Every Rule 56 movant bears the burden of establishing the absence of any genuine issue of material fact (Celotex Corp. v. Catrett, 477 U.S. 317, 322-23 (1986)).[2] For that purpose

[1] All further references to Title 35's provisions will simply take the form "Section --," omitting the prefatory "35 U.S.C. §."

[2] At the summary judgment stage, of course, Celsis need not "establish" or "show" or "prove" anything to defeat LTC's motion, but must merely demonstrate that a genuine issue of (continued)

courts consider the evidentiary record in the light most favorable to nonmovants and draw all reasonable inferences in their favor (Lesch v. Crown Cork & Seal Co., 282 F.3d 467, 471 (7th Cir.2002)). But a nonmovant must produce more than "a mere scintilla of evidence" to support the position that a genuine issue of material fact exists (Wheeler v. Lawson, 539 F.3d 629, 634 (7th Cir.2008)) and "must come forward with specific facts demonstrating that there is a genuine issue for trial" (id.). Ultimately summary judgment is warranted only if a reasonable jury could not return a verdict for the nonmovant (Anderson v. Liberty Lobby, Inc., 477 U.S. 242, 248 (1986)). What follows is a summary of the facts,[3] viewed in the light most favorable to nonmovant Celsis.

Whether a patent is valid under either Section 101 or Section 112 is a question of law (Fort Properties, Inc. v. Am. Master Lease LLC, 671 F.3d 1317, 1320 (Fed. Cir. 2012) as to Section 101 and Microprocessor Enhancement Corp. v. Texas Instruments Inc., 520 F.3d 1367, 1374 (Fed. Cir. 2008) as to Section 112). And because there is a statutory presumption of patent validity, LTC must prove invalidity by clear and convincing evidence (Trimed, Inc. v. Stryker Corp., 608 F.3d 1333, 1340 (Fed. Cir. 2010)), at least with respect to Section 112. Although a recent concurring opinion in Ultramercial, Inc. v. Hulu, LLC, 772 F.3d 709, 720-21 (Fed. Cir.

(footnote continued)
material fact exists. This opinion employs the quoted terms only because the cited cases use that terminology, but it imposes on Celsis the lesser burden described earlier in this footnote.

[3] This District Court's LR 56.1, adopted to implement Rule 56, requires parties to submit evidentiary statements and responses to such statements to highlight which facts are disputed and which facts are agreed upon. This opinion cites to LTC's LR 56.1 statement as "LTC St. ¶ ," to Celsis' LR 56.1 statement as "C. St. ¶" and to Celsis' response to LTC's LR 56.1 statement as "C. Resp. ¶ --" (oddly, LTC has chosen not to file any response to Celsis' LR 56.1 statement). Where a party's response does not provide a different version of the facts than the original statement, this opinion cites only that original statement.

2014) has suggested that no such presumption attaches to patent eligibility -- and hence to patent validity -- under Section 101, this opinion need not pause to consider that possibility, for the well-settled facts compel a finding of invalidity under Section 101 regardless of which standard this Court applies.

Facts

United States Patent No. 7,604,929 ("the '929 Patent," LTC St. Ex. A) protects several variants on a claimed process for cryogenically freezing hepatocytes (a type of liver cell). Hepatocytes are useful for a variety of testing, diagnostic and treatment purposes (id. at col. 5 ll. 26-27), but before Celsis' innovation there were significant problems with using hepatocytes for those purposes (id. at col. 2 l. 22 to col. 3 l. 67). First, hepatocytes have a short lifespan, and their supply is inconsistent because it is dependent upon the availability of liver cells (id. at col. 2 ll. 30-32). Moreover, to test drugs accurately researchers prefer to use pools of hepatocytes from many different liver donors (id. at col. 3 ll. 33-49). But until Celsis's contribution, pooling hepatocytes from different donors was difficult due to the erratic supply and short lifespan of the cells (id. at col. 3 ll. 49-52).

Accordingly scientists sought ways to cryopreserve hepatocytes for later use (id. at col. 2 ll. 36-40), but both scientists and researchers found that cryopreservation significantly decreased cell viability (id. at col. 3 ll. 5-8). Prevailing wisdom therefore taught that cells could be frozen only once and then had to be used or discarded (LTC St. Ex. B ["Hardy Dep."] 129:2-129:6). That severely limited the creation of pooled hepatocyte products ('929 Patent col. 3 ll. 30 to col. 4 ll. 6).

Essentially the method taught in the '929 Patent shows that cells can be frozen and refrozen without losing significant cell viability, so that pooled hepatocyte products are far more

readily attained ('929 Patent col. 3 l. 61 to col. 4 l. 6). That process can be summarized as (1) thawing previously frozen cells, (2) separating nonviable cells from viable ones using "density gradient fractionation (especially Percoll density centrifugation)" and (3) refreezing the cells (id. at col. 4 ll. 38-50). With nonviable cells separated out, the resulting cell preparation contains a higher concentration of viable cells that can be subjected to repeated cryopreservation and thawing for drug testing and other purposes (C. Resp. ¶¶ 14-16; '929 Patent col. 9 l. 61 to col. 10 l. 67). Co-inventor James Hardy stated that the enhanced viability of the solution is attributable to a change in ratios: Reducing the raw number of nonviable cells in the solution necessarily increases the ratio of viable cells to overall cells in the solution. Hardy could not confirm that the process improved the health of any one individual viable cell (C. Resp. ¶¶ 14-16), though he did note that dead and dying cells can release harmful substances into cell solutions, so that removing nonviable cells benefits the population of viable cells (C. Resp. Ex. 37 49:25-50:6).

Here is the relevant language of Claim 1 ('929 Patent at col. 19 l. 55 to col. 20 l. 20), which is also representative of the other claims at issue:

> 1. A method of producing a desired preparation of multi-cryopreserved hepatocytes, said hepatocytes, being capable of being frozen and thawed at least two times, and in which greater than 70% of the hepatocytes of said preparation are viable after the final thaw, said method comprising:
>
> (A) subjecting hepatocytes that have been frozen and thawed to density gradient fractionation to separate viable hepatocytes from non-viable hepatocytes,
>
> (B) recovering the separated viable hepatocytes, and
>
> (C) cryopreserving the recovered viable hepatocytes to thereby form said desired preparation of hepatocytes without requiring a density gradient step after thawing the hepatocytes for the second time, wherein the hepatocytes are not plated between the first and second cryopreservations, and wherein greater than 70% of the hepatocytes of said preparation are viable after the final thaw.

As for the process involved in twice cryopreserving hepatocytes, it duplicated the technique used for single cryopreservation: subjecting the cells to density gradient fractionation (to sort out the viable from the nonviable cells) and then freezing them (Hardy Dep. 129:18-130:6; LTC St. ¶¶ 8-12).

Armed with the discovery that cells were capable of being twice frozen, Celsis built on that prior art method by repeating it. As Hardy himself admitted in his deposition, the critical advance was the discovery that cells could be frozen more than once and still retain viability ((Hardy Dep.. 127:9-131:21) (emphasis added)):

> A: My recollection, I haven't thought about this in awhile, but I think initially we just proved that you could twice freeze the cells and still have viable cells. And then we added Percoll, later, because we wanted the viable cell count to be higher, you know, what our standard specification was above 70 percent when they were thawed, because we saw ad- -- you know, a loss somewhere.
>
> Q: And you knew from prior experience that if you had a -- the -- that from freezing cells that you were going to get some loss?
>
> A: We were going to get some loss.
>
> Q: So you wanted to bump the number up before you froze it?
>
> A: Froze it again.
>
> Q: And the common procedure that you had known from the time you started with the company for bumping up the viability of hepatocyte cells is a Percoll centrifugation; correct?
>
> A: Mm-hmm. Mm-hmm.
>
> Q: Okay.
>
> A: Yes.
>
> Q: That's why you did it, and you got the outcome that you expected that you would get; correct?

A: The unexpected outcome was that you would get any activity of cytochromes at all on a fro- -- twice frozen cell, so that was the unexpected outcome, it would be expected if you would have viable cells, if they perform the way that they're supposed to, the unexpected outcome was that cells twice frozen behaved like cells that were once frozen.

Q: Behaved in terms of their enzyme operation; correct?

A: The metabolic activities as we measured them, yes.

Q: Okay. But you weren't surprised that you ended up with viable cells?

A: No, we were very surprised . . .
The industry taught me that you can only freeze them once, then they were no longer any good, that's what I learned from Paul Silber, who was the expert in hepatocytes, and -- and the other literature, that you can only freeze them one time. . .

Q: So what you -- what you did was you took cells that had been made -- had been isolated from the liver, and run through a Percoll centrifugation, and then frozen with the typical cryopreservation methodology, you took those cells now as your starting point instead of starting with a fresh liver, and you repeated the exact same process that had been used before in terms of cleaning up the cells and increasing their viability through a Percoll, losing 70 percent, 30 -- you know, 50 to 70 percent of the cells in the process, and running the same cryopreservation technique and ended up with product that got acceptable viability; correct?

A: Yes. . .

Q: Now, the process by which [you increased the percentage of cell viability] . . . there's nothing in that process that brings the cells back to life, what happens is you end up essentially playing with the denominator; isn't that true? You eliminate 50 to 70 percent of the cells that are in there and then that gets you a -- a higher viability; isn't that right?

A: Yes. You separate the non-viable cells from the viable cells.

All of the remaining claims in the '929 Patent are variants on that process but go on to specify that Percoll be used for density gradient fractionation (Claim 2), that the hepatocytes are selected from a specified group of mammals including humans (Claim 3) or are human (Claim 4), that 80% of the hepatocytes of the resulting preparation are viable (Claim 9) and

constitute a "pooled preparation of hepatocytes of multiple sources" (Claim 5) that are either pooled according to gender, race or state of health (Claims 6 and 11) or have a specific type or level of some type of metabolic activity (Claims 7 and 8) (LTC St. ¶ 18). It is undisputed that a determination that Claim 1 is invalid dooms all of those dependent claims.

It is important to note that the '929 Patent specifies that as a result of the patented method the resulting cell preparation should demonstrate at least 70% or 80% viability, with the patent specifying that scientists should measure viability using the "Trypan Blue exclusion" method (LTC St. ¶ 21). That test is known to yield variable measurements, though the parties dispute precisely how variable: LTC asserts the variability is about 50%, while Celsis asserts that it is 15% or less (C. Resp. ¶¶ 22-24). In any case, the Trypan Blue exclusion method was the standard industry test: It was used by many if not most customers, and LTC itself uses that method to measure viability (C. St. ¶¶ 1-3). Although there is an alternative and less variable method of testing hepatocyte viability (using a Guava Counter), Celsis claims that method is impractical for hepatocyte testing, so that the Trypan Blue exclusion method was the best measurement technique for the industry (C. St. ¶ 5; Dryden Decl. ¶ 17).

Procedural Posture

Celsis filed this action for infringement of its '929 Patent in June 2010, and this litigation has since subjected the patent's validity and scope to repeated examination. Initially this Court determined that Celsis had a likelihood of success on the merits when it granted Celsis' motion for a preliminary injunction back in September 2010, a decision that the Federal Circuit then affirmed on January 9, 2012 (664 F.3d 922, 924, 926 (Fed. Cir. 2012)). Next an ex parte reexamination by the Patent and Trademark Office ("PTO") separately reconfirmed the '929

Patent's validity (though it canceled one claim not at issue here) on February 28, 2012 (Ex. B to Strom Decl.).

LTC has since engineered around the patent to create pooled hepatocyte products using a newly developed elutriation process that this Court ultimately determined did not infringe the '929 Patent (21 F. Supp. 3d 960, 962-63 (N.D. Ill. 2014)). That ultimate holding reconfirmed this Court's March 24, 2011 opinion (995 F.2d 855) that had reached the same conclusion after another several-day evidentiary hearing and had therefore rejected Celsis' effort to obtain a second preliminary injunction, this time targeting LTC's method as an asserted infringement of the '929 Patent. And that 2011 determination by this Court was also affirmed by the Federal Circuit on October 21, 2011, this time in a per curiam opinion that adopted this Court's reasoning:

> After full de novo review of the record, the parties' briefs, and counsels' arguments, and for the reasons articulated in the district court's decision, we agree with, and thus adopt, the district court's construction of "density gradient fractionation" and without requiring a density gradient fractionation step after thawing the hepatocytes for a second time.[2] In light of these claim constructions, the district court did not abuse its discretion in concluding that a showing of literal infringement is not likely.
>
> ---
>
> [2] We find that the district court carefully considered the language of the claims, the specification and prosecution history, and the testimony of the parties' witnesses in reaching its conclusions regarding the proper construction of the claims and, thus, remained true to our guidance in Phillips v. AWH Corp., 415 F.3d 1303, 1313-19 (Fed. Cir. 2005) (en banc).

But the abundance of opinions (both published and unpublished) and of other rulings during the active course of this litigation (which as of this writing has spawned no fewer than 428 docket entries at the District Court level, encompassing both the litigants' filings and those court actions) has never called upon either this Court or the Federal Circuit to address the issues

or analysis that are now before this Court under the stimulus of the Supreme Court's more recent and more intensive analysis of some fundamental patent law principles and the Federal Circuit's application of those principles.[4]

Most recently, in an effort to narrow the issues in the litigation, the parties have stipulated that if the '929 Patent is deemed valid, LTC infringed at least one of the patent's claims when it manufactured certain lots (Joint Stipulation, ECF No. 418). Thus what are now pending for decision are two motions by LTC -- one challenging the validity of the patent and a second seeking to limit any damages to which Celsis is entitled

Patent Validity Under Section 101

LTC argues that the '929 patent is invalid under Section 101 because it lacks a sufficiently inventive step to constitute patentable subject matter. Section 101 provides that "[w]hoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent." Excepted from patentability, however, are "laws of nature, physical phenomena, and abstract ideas" (Bilski v. Kappos, 561 U.S. 593, 601 (2010), quoting Diamond v. Chakrabarty, 447 U.S. 303, 309 (1980)). Patent law makes a conscious choice not to "inhibit further discovery by improperly tying up the future use of these building blocks of human ingenuity" (Alice Corp. Pty. v. CLS Bank Int'l, 134 S. Ct. 2347, 2354 (2014) (internal quotation marks omitted)).

[4] As the ensuing text reflects, it is the Supreme Court's in-depth analysis in the 2012 decision in the Mayo case and the 2014 decision in the Alice case (both of those are unanimous decisions) that provides the road map for any determination of the validity of a process patent whose springboard is some law of nature -- and, of course, neither of those cases had been decided when this Court rendered its 2010 decision finding the '929 Patent valid en route to its grant of a preliminary injunction in Celsis' favor and when the Federal Circuit issued its opinion at the very beginning of 2012 affirming that decision.

While distinguishing between patentable application and unpatentable principle makes good sense in the abstract, drawing that line has proved far more difficult for the courts, especially when it comes to process patents (see Parker v. Flook, 437 U.S. 584, 589 (1978), and more recently see also, e.g., Diamond v. Diehr, 450 U.S. 175 (1981); Mayo Collaborative Servs. v. Prometheus Labs., Inc., 132 S. Ct. 1289 (2012); Alice, cited above; Univ. of Utah Research Found. v. Ambry Genetics Corp., 774 F.3d 755 (Fed. Cir. 2014)). Because a patentable process by definition yields a prescribed result, it functions in some sense as a "law" -- the law being that steps 1 through "n" always yield specified outcome "x." So deciding whether a procedure is one deserving patent protection, or whether it is effectively locking up something that should instead remain free for all to use, is a slippery slope. And precisely because, on some level, "all inventions . . . embody, use, reflect, rest upon, or apply" unpatentable subject matter, the Supreme Court has repeatedly cautioned courts to "tread carefully in construing this exclusionary principle lest it swallow all of patent law" (Alice, 134 S. Ct. at 2354).

Recent Supreme Court decisions Mayo and Alice have shed new light on Section 101 patent validity. Mayo laid the groundwork for the precise two-part test articulated in Alice, 134 S. Ct. at 2355: First a court must "determine whether the claims at issue are directed to one of those patent-ineligible concepts."[5] If so, the court must then proceed to the second step and inquire "[w]hat else is in there in the claims before us?" (id.) At that point the court must "consider the elements of each claim both individually and as an ordered combination to

[5] Because that and all of the other quotations in this and the next paragraphs of the text were drawn from Mayo in the Alice opinion, this opinion will speak (for example) of the "Mayo-Alice step 1" (just as in a different context this Court customarily refers to the "Twombly-Iqbal canon").

determine whether the additional elements transform the nature of the claim into a patent-eligible application" (id., internal quotation marks omitted).

That second step has been characterized in Alice, id. (emphasis in original and internal quotation marks omitted) as the "search for an inventive concept -- i.e., an element or combination of elements that is sufficient to ensure that the patent in practice amounts to significantly more than a patent upon the [ineligible concept] itself." Though the caselaw identifying what constitutes an "inventive concept" is still young, as is made clear by the preceding language, the core concern motivating that second step is one of preemption.

Of course "to transform an unpatentable law of nature into a patent-eligible application of such a law, one must do more than simply state the law of nature while adding the words 'apply it.'" (Mayo, 132 S. Ct. at 1294 (emphasis in original)). There the Supreme Court invalidated a process patent for calibrating drug dosages, where the process basically consisted of nothing more than administering a drug to patients measuring the concentration of relevant metabolites in those patients and then adjusting drug dosage up or down based upon how those metabolite concentrations correlate with drug levels (id. at 1295). Effectively the patent claimed ownership of a natural law -- the natural law being how metabolite concentrations correlated with drug dosages -- while each of the remaining "steps" in the process was necessary to apply that law (id. at 1297-98). So even though drafted as a process, the patent was really nothing more than a statement of a law of nature and an instruction to apply it (id. at 1298).

Importantly, patent law's prohibition on unpatentable subject matter cannot be circumvented by simply adding insignificant postsolution activity or by limiting the use of a formula to a specific technological environment (Mayo, 132 S. Ct. at 1294). Similarly, the addition of "well-understood, routine, conventional activity already engaged in by the scientific

community" is insufficient to transform an ineligible concept into patentable subject matter (id. at 1298). At the same time, the combination of routine steps may well yield a process that on the whole warrants patent protection (see Diamond v. Diehr, 450 U.S. 175, 188 (1981)).

Two Supreme Court cases, Parker and Diamond v. Diehr, are often contrasted to illustrate the distinction between patentable and unpatentable subject matter. Parker, 437 U.S. at 594 found a method for calculating alarm limit values unpatentable where the method consisted of three steps: measurement of different variables, calculation of a new limit by plugging those measurements into a formula and adjustment of the actual alarm limit based on the updated calculations (id. at 585). Though the patent did not preempt every single application of the formula (id. at 586), nothing about the process other than the unpatentable mathematical algorithm was new or useful (id.at 591).

Shortly thereafter in a case involving a similar set of facts, Diamond v. Diehr reasoned to the contrary. At issue there was a process for curing rubber that involved use of a long-known formula, the Arrhenius equation, to calculate the appropriate cure time (450 U.S. at 177-79, 192-93). As in Parker, the patented method involved just a few simple steps: installing rubber in a press, closing the mold, continuously measuring the temperature inside the mold, feeding the measured temperature information to a computer to recalculate an updated cure time and automatically opening the press at the proper time (id. at 187). Nonetheless the Supreme Court upheld the patent. In contrast to Parker, absent from the facts in Diamond v. Diehr was any suggestion that "all these steps, or at least the combination of those steps, were in context obvious, already in use, or purely conventional" -- instead the process was "an inventive application" of an otherwise unpatentable formula (Mayo, 132 S. Ct. at 1299). Thus the patentees did not "seek to pre-empt the use of [the] equation" but sought "only to foreclose from

others the use of that equation in conjunction with all of the other steps in their claimed process" (id.).

LTC argues that the '929 Patent is invalid because the claims (1) are directed toward unpatentable subject matter and (2) fail to incorporate any inventive concept. This Court agrees.

Applying Mayo-Alice step 1, this Court concludes that the patent is directed to an ineligible law of nature: the discovery that hepatocytes are capable of surviving multiple freeze-thaw cycles.[6] Though the parties cite limited caselaw detailing the meaning of "directed to" patent-ineligible subject matter, the PTO recently issued its 2014 Interim Guidance on Patent Subject Matter Eligibility, 79 C.F.R. 74622 (Dec. 16, 2014), currently subject to public comment, that defines Alice step 1 as requiring that a law of nature, natural phenomenon or abstract idea be "recited (*i.e.*, set forth or described) in the claim." In this instance Claim 1 of the '929 Patent claims "a method of producing a desired preparation of multi-cryopreserved hepatocytes, said hepatocytes, being capable of being frozen and thawed at least two times," and the claimed method then outlines a process for freezing the cells twice. Clearly, therefore, the patent recites the natural law that certain hepatocytes are capable of being frozen and thawed more than once. Each of the other claims is dependent on that method and so also incorporates the same recitation.

Applying Mayo-Alice step 2, this Court further agrees that the patented process lacks the requisite inventive concept. One of the inventors, James Hardy, said that "the unexpected

---

[6] LTC further argues that the patent is directed toward "natural phenomena" -- hepatocytes. However, because the Court finds that the patent is directed toward a patent-ineligible law of nature, this opinion does not further address whether the process patent is also directed toward "natural phenomena." It likewise need not treat with the parties' dispute as to any continued applicability of the hoary decision in Funk Brothers Seed Co. v. Kalo Innoculant Co., 333 U.S. 127 (1948).

outcome was that cells twice frozen behaved like cells that were once frozen" -- unquestionably a natural characteristic of the hepatocytes, though no one may have remarked it before -- and it is undisputed that upon making that discovery Hardy reapplied a well-understood freezing process.

Celsis argues that repetition of that already-well-established process itself constitutes the requisite inventive concept because prior art taught away from multiple freezings. But in determining whether the process warrants patent protection, we ask "what else" is in the patent beyond the patent-ineligible concept. Here the answer is not much. This patent amounts to a straightforward application of the truth that hepatocytes are inherently capable of surviving multiple freeze-thaw cycles.[7] In light of that determination this Court need not separately analyze whether the process meets the "machine-or-transformation" test, which is an "important and useful clue to patentability" but does not "trump[ ] the law of nature exclusion" (Mayo, 132 S. Ct. at 1303 (emphasis in original and internal quotation marks omitted)).

---

[7] At the District Court level Ameritox, Ltd. v. Milennium Health, L.L.C., 13-CV-832-wmc, 2015 U.S. Dist. LEXIS 19665 (W.D. Wis. Feb. 18, 2015) has stated that "if inventors engage in activities that run counter to scientific thought, those activities can hardly be considered conventional under § 101." Even putting to one side the truism that, as our Court of Appeals regularly (and properly) reminds us, District Court opinions carry no precedential weight, that case and the context for the court's decision were factually very different from the case at hand. Ameritox, id. at *18-24, *58 upheld a patent for a method of urine testing that improved the medical community's ability to monitor a patient's compliance with prescribed treatment regimens. That method involved normalizing a patient's urine sample by determining the metabolite/creatinine ratio and then comparing the normalized test results to a set of known normative data collected from other patients who were adherent to the proper treatment regimen (id. at *18-19). Thus the process as a whole satisfied Mayo-Alice step 2 because it creatively marshaled techniques that no scientist would have thought to apply to the particular field at the time. No prior art reference suggested both normalizing creatinine levels and comparing one patient's normalized data to that of other individuals to monitor treatment compliance -- and prior art taught that creatinine normalization was unreliable and that blood testing was preferable to urine testing (id. at *78). Here by contrast the combination of steps in the '929 Patent directly follows from the discovery of a law of nature: that hepatocytes are capable of surviving multiple freeze-thaw cycles -- and the patent directs the employment of methods that were routinely used in the prior art for precisely the same purpose of cryogenization to preserve such cells.

It is true that Diamond v. Diehr cautioned against dissecting claims into their constituent elements and has therefore instructed courts to construe claims as a whole. At the same time, Alice and Mayo demand that courts look beyond the natural law itself and clearly dictate that conventional steps, or the addition of insignificant postsolution activity, are insufficient to transform the unpatentable into the patentable. Here the process outlined for the second freezing is postsolution activity, implemented with entirely conventional methods.

What has been said to this point has taken care not to trench on Sections 102 and 103, which govern novelty and nonobviousness. Those sections are concerned with whether an invention is novel or nonobvious in light of the prior art -- and as Mayo, 132 S. Ct. at 1304 remarked, "§§ 102 and 103 say nothing about treating laws of nature as if they were part of the prior art when applying those sections."

Because claim 1 fails under the Mayo-Alice test, the other claims -- which the parties agree simply add well-known and conventional concepts -- also fail. Indeed, neither party has addressed those claims separately in its briefing.

Finally it is worth noting that this case is somewhat unique in that, although the '929 Patent lacks an inventive concept, it is more narrowly drawn than the patents at issue in Mayo and Alice because it does not lock up the natural law in its entirety. As stated earlier, LTC has already managed to engineer around the patent by using a different mechanism for sorting viable from nonviable cells called elutriation, though the effectiveness of that method -- and perhaps of other alternatives -- is subject to dispute in LTC's damages motion (see LTC's Resp. to Celsis' LR 56.1 St. ¶ 6 on the damages issue, Dkt. 409). In any event Univ. of Utah, 774 F.3d at 764 n.4 recently reasoned that the "preemptive nature of the claims" at issue in that case was "not ameliorated" by virtue of the fact that there might have been other routine ways to get around the

patent -- if patent law were to permit a lock on a narrow albeit routine combination of steps, "different combinations of other routine steps" (id.) would also be patent-eligible.

Put another way, if one were allowed to own a slice of the preemptive pie, that would pave the way for multiple others to claim the rest of that pie. Such a result would clearly run counter to the teaching and purpose of Mayo and Alice. Hence this Court adopts the Univ. of Utah reasoning here.[8]

Patent Validity Under Section 112

Section 112(b) requires that a patent specification "conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention." Until recently a patent satisfied that definiteness requirement before the Federal Circuit so long as it was not "insolubly ambiguous" (Nautilus, Inc. v. Biosig Instruments, Inc., 134 S. Ct. 2120, 2124 (2014)). Last year Nautilus, id. (emphasis added) replaced that standard with a less lenient one under which "a patent is invalid for indefiniteness if its claims, read in light of the specification delineating the patent, and the prosecution history, fail to inform, with reasonable certainty, those skilled in the art about the scope of the invention." That standard "mandates clarity, while recognizing that absolute precision is unattainable" (id. at 2129) -- and "the certainty which the law requires in patents is not greater than is reasonable, having regard to their subject-matter" (id., quoted from Minerals Separation, Ltd. v. Hyde, 242 U.S. 261, 270 (1916)). Importantly, indefiniteness must be assessed from the perspective of someone skilled in the art at the time of the patent filing (id. at 2128).

[8] See also 1 Donald Chisum, Chisum on Patents § 1.03[2][f] at 1-138.27 (2014), noting that Alice's two-part test does not separately require courts to examine whether the patent disproportionately ties up a law of nature.

LTC also argues that the '929 Patent is indefinite as a matter of law because the Trypan Blue Exclusion method is so inherently variable, varying as much as 50%. Celsis counters that the variance is some 15% or less and that, because it is the standard industry test -- used even by LTC -- it cannot possibly fail to inform those skilled in the art of the metes and bounds of the patent "with reasonable certainty." Although it seems that Celsis has the better of it at the summary judgment stage (each of the cases sought to be relied on by LTC is inapposite), the Section 112(b) issue need not be addressed (let alone resolved). What has gone before has dispatched the '929 Patent under Section 101, and that is enough.

Conclusion

Because the patent is invalid under Section 101, this Court grants LTC's Motion for Summary Judgment of Patent Invalidity (Dkt. 335). That causes LTC's Motion To Limit Damages to a Reasonable Royalty on LTC's Accused Sales (Dkt. 337) to be denied as moot.

Milton I. Shadur
Senior United States District Judge

Date: March 13, 2015

IN THE UNITED STATES DISTRICT COURT
FOR THE NORTHERN DISTRICT OF ILLINOIS
EASTERN DIVISION

CELSIS IN VITRO, INC., Plaintiff,	)	
v.	)	Case No. 10 C 4053
CELLZDIRECT, INC., a Delaware Corporation and wholly-owned subsidiary of **INVITROGEN CORPORATION**; and **INVITROGEN CORPORATION**, a Delaware Corporation, Defendants.	)	

SUPPLEMENT TO MEMORANDUM OPINION AND ORDER

Because the parties' submissions in support of and in opposition to the entry of summary judgment in favor of defendants (this supplement, like this Court's March 13 memorandum opinion and order (the "Opinion"), collectivizes defendants as "LTC" for convenience) were filed under seal, the Opinion has also been filed under seal pending input from the litigants' counsel as to any possible redactions or other handling. In the meantime this Court has taken a final look at the Opinion and is left with the sense that its discussion of technical issues and of the changes marked by the Mayo-Alice approach to patent eligibility may have inadvertently obscured a portion of the forest for the trees.

Accordingly, in the interest of clarity, this supplement to the Opinion is issued to provide a more brief summary of its fundamental holding. And to that end this Court believes that it cannot readily improve on this presentation in the Introduction section of LTC's summary judgment motion at its pages 1 and 2 (Dkt. 335):

> As to § 101, all remaining claims of the '929 patent fail because they consist of nothing more than an observed law of nature combined with the application of

> routine, conventional steps. Specifically, those claims merely recite the natural fact that, in a normal population of hepatocytes (themselves nothing more than an isolated product of nature), some sub-population is capable of surviving the process of being frozen and thawed at least two times and some sub-population is not. The remaining claim elements consist of the application of only well-understood, routine, and conventional cell separation and cryopreservation steps admittedly in common use long before the time of the claimed inventions.
>
> '929 patent inventors Dryden and Hardy readily admit that the processing steps in the claimed methods do nothing to alter the physical properties of the hepatocyte cells put through that process; instead, the claims merely reflect the natural phenomenon that some of those cells are inherently capable of surviving multiple freeze-thaw cycles and others are not. Dryden and Hardy likewise admit that their patent claims disclose no new approaches to isolating, freezing, thawing, "reformulating," or refreezing the cells. All of the claimed steps are admittedly old and conventional. Indeed, the sole "discovery" of value claimed by the inventors is that some hepatocytes can be frozen multiple times (using known techniques) and remain viable, and those hepatocytes can be separated from those that do not remain viable (also using conventional means). That the "discovery" facilitated the development of a commercially successful product is of no moment. Discovery of a natural law simply does not qualify as patentable subject matter; nor does any other part of the '929 patent's claims display the requisite inventiveness to satisfy § 101.

It is for that fundamental analysis of the '929 Patent's absence of patent eligibility, which clearly includes the hepatocyte cell separation process employed as part of the cryopreservation technique described in the patent, that this Court (1) has granted summary judgment in LTC's favor and (2) has determined that a final judgment should be entered dismissing this action with prejudice. This Court so orders.

Milton I. Shadur
Senior United States District Judge

Date: March 16, 2015

IN THE UNITED STATES DISTRICT COURT
FOR THE
NORTHERN DISTRICT OF ILLINOIS

Celsis In Vitro, Inc.
Plaintiff(s)

v.

CellzDirect, Inc. et al
Defendant(s)

Case No. 10 C 4053

JUDGMENT IN A CIVIL CASE

Judgment is hereby entered (check appropriate box):

□ in favor of plaintiff(s)
and against defendant(s)
in the amount of $,

which □ includes pre–judgment interest.
□ does not include pre–judgment interest.

Post-judgment interest accrues on that amount at the rate provided by law from the date of this judgment.

Plaintiff(s) shall recover costs from defendant(s).

□ in favor of defendant(s)
and against plaintiff(s)
.

Defendant(s) shall recover costs from plaintiff(s).

☒ other: Final judgment is entered dismissing this action with prejudice.

This action was *(check one)*:

□ tried by a jury with Judge presiding, and the jury has rendered a verdict.
□ tried by Judge without a jury and the above decision was reached.
☒ decided by Judge Milton I. Shadur.

Date: 3/16/2015

Thomas G. Bruton, Clerk of Court

Carol Wing,, Deputy Clerk

IN THE UNITED STATES DISTRICT COURT
FOR THE
NORTHERN DISTRICT OF ILLINOIS

Celsis In Vitro, Inc.
Plaintiff(s)

v.

CellzDirect, Inc. et al
Defendant(s)

Case No. 10 C 4053

AMENDED JUDGMENT IN A CIVIL CASE

Final judgment is hereby entered (check appropriate box):

□ in favor of plaintiff(s)
and against defendant(s)
in the amount of $,

which □ includes pre–judgment interest.
□ does not include pre–judgment interest.

Post-judgment interest accrues on that amount at the rate provided by law from the date of this judgment.

Plaintiff(s) shall recover costs from defendant(s).

☒ in favor of defendant(s)CellzDirect, Inc. and Invitrogen Corporation
and against plaintiff(s)Celsis In Vitro, Inc.
. dismissing this action with prejudice.

Defendant(s) shall recover costs from plaintiff(s).

□ other:

This action was *(check one)*:

□ tried by a jury with Judge presiding, and the jury has rendered a verdict.

□ tried by Judge without a jury and the above decision was reached.

☒ decided by Judge Milton I. Shadur.

Date: 4/3/2015

Thomas G. Bruton, Clerk of Court

Carol Wing,, Deputy Clerk

US007604929B2

(12) **United States Patent**
Dryden et al.

(10) **Patent No.: US 7,604,929 B2**
(45) **Date of Patent: Oct. 20, 2009**

(54) **CELLULAR COMPOSITIONS AND METHODS FOR THEIR PREPARATION**

(75) Inventors: **Daniel Dryden**, Westminter, MD (US); **James Hardy**, Ijamsville, MD (US)

(73) Assignee: **In Vitro Technologies, Inc.**, Baltimore, MD (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 318 days.

(21) Appl. No.: **11/110,879**

(22) Filed: **Apr. 21, 2005**

(65) **Prior Publication Data**

US 2005/0239042 A1 Oct. 27, 2005

(51) **Int. Cl.**
A01N 1/00 (2006.01)
C12N 5/00 (2006.01)
C12N 5/08 (2006.01)

(52) **U.S. Cl.** **435/1.1**; 435/1.3; 435/370; 435/374; 435/375

(58) **Field of Classification Search** 435/1.1, 435/1.3, 370, 374, 375
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,795,711	A	8/1998	Mullon et al.
5,895,745	A	4/1999	Chandler et al.
6,136,525	A	10/2000	Mullon et al.
6,759,245	B1	7/2004	Toner et al.
2002/0039786	A1	4/2002	Reid et al.
2003/0134418	A1	7/2003	Mitaka

FOREIGN PATENT DOCUMENTS

EP	0834252 B	4/1998
WO	WO92/12722	8/1992
WO	WO/0153462	7/2001
WO	WO03/105663	12/2003
WO	WO2004/009766	1/2004
WO	WO2005/000376	1/2005

OTHER PUBLICATIONS

Ostrowska et al., Cell and Tissue Banking, 2000, 1: 55-68.*
Shibata et al., Drug Metabolism and Disposition, 2002, 30: 892-896.*
Oehninger et al., Mol Cell Endocrinol, 2000, 169: 3-10.*
Hengstler et al., Drug Metab. Rev., 2000, 32: 81-118, Abstract.*
Arikura et al., UW solution: a promising tool for cryopreservation of primarily isolated rat hepatocytes, 9 J. Hepatobiliary Pancreat. Surg. 742-749 (2002).
Marsh et al., Hypothermic Preservation of Hepatocytes III Effects of Resuspension Media on Viability after up to 7 Days of Storage, 13(3) Hepatology 500-508 (1991).
Smrzova et al., Optimisation of Porcine Hepatocyte Cryopreservation by Comparison of Viability and Enzymatic Activity of Fresh and Cryopreserved Cells, 70 Acta Vet. Bmo. 141-147 (2001).
Vincent et al., Adjustment of the Osmolality of Percoll for the Isopycnic Separation of Cells and Cell Organelles, 141 Analytical Biochemistry 322-328 (1984).
Adams, R.M. et al. (1995) "*Effective Cryopreservation And Long-Term Storage Of Primary Human Hepatocytes With Recovery Of Viability, Differentiation, And Replicative Potential*," Cell Transplant. 4(6):579-586.
Alexandre, E. et al. (2002) "*Cryopreservation Of Adult Human Hepatocytes Obtained From Resected Liver Biopsies*," Cryobiology 44:103-113.
Anand, A.C. (1996) "*Bioartificial Livers: The State Of The Art*," Trop Gastroenterol. 17(4):197-198, 202-211.
Berry, M.N. et al. (1992) "*Techniques for* Pharmacological And *Toxicological Studies With Isolated Hepatocyte Suspensions*," Life Sci. 51(1):1-16.
Burlina, A.B. (2004) "*Hepatocyte Transplantation For Inborn Errors Of Metabolism," J. Inherit. Metab. Dis.* 27(3):373-83.
Chan, C. et al. (2004) "*Hepatic Tissue Engineering For Adjunct And Temporary Liver Support: Critical Technologies," Liver Transpl.* 10(11):1331-1342.
Chesne, C. et al. (1993) "*Viability And Function In Primary Culture Of Adult Hepatocytes From Various Animal Species And Human Beings After Cryopreservation*," Hepatology 18(2):406-414.
Coundouris, J.A. et al. (1993) "*Cryopreservation Of Human Adult Hepatocytes For Use In Drug Metabolism And Toxicity Studies*," Xenobiotica. 23(12):1399-1409.
Diener, B. et al. (1993) "*A Method For The Cryopreservation Of Liver Parenchymal Cells For Studies Of Xenobiotics*," Cryobiology 30(2):116-127.
Dou, M. et al. (1992) "*Thawed Human Hepatocytes In Primary Culture*," Cryobiology 29(4):454-69.
Fox, I.J. et al. (2004) "*Hepatocyte Transplantation," Am. J. Transplant.*4 Suppl. 6:7-13.
Fukuda, J. et al. (2004) "*Hepatocyte Organoid Culture In Elliptic Hollow Fibers To Develop A Hybrid Artificial Liver*," Int J Artif Organs. 27(12):1091-1099.
Gan, J.H. et al. (2005) "*Hybrid Artificial Liver Support System For Treatment of Severe Liver Failure*," World J Gastroenterol. 11(6):890-894.
Gomez-Lechon, M.J. et al. (2004) "*Human Hepatocytes In Primary Culture: The Choice To Investigate Drug Metabolism in Man*," Curr Drug Metab. 5(5):443-462.
Guillouzo, A. et al. (1986) "*Isolated and Cultured Hepatocytes*," Paris: les Editions INSERM and London: John Libbey Eurotext).
Hewitt, N.J. et al. (2004) *Cryopreserved Rat, Dog and Monkey Hepatocytes: Measurement Of Drug Metabolizing Enzymes In Suspensions And Cultures*, Hum Exp Toxicol. 23(6)307-316.
Horslen, S.P. (2004) "*Hepatocyte Transplantation," Transplantation* 77(10):1481-1486.
Houle, r. et al. (2003) "*Retention Of Transporter Activities in Cryopreserved, Isolated Rat Hepatocytes*," Drug Metab. Disposit. 31(4):447-451.
Kasai, s. et al. (1993) "*Large Scale Cryopreservation of Isolated Dog Hepatocytes," Cryobiology* 30:1-11.

(Continued)

Primary Examiner—Ileana Popa
(74) *Attorney, Agent, or Firm*—Loeb & Loeb LLP

(57) **ABSTRACT**

The present invention relates to novel cell (e.g., hepatocyte, etc.) compositions and methods for their preparation and use. In particular, the invention concerns methods of processing preparations of such cells so as to permit their repeated cryopreservation and thawing while retaining substantial viability. The invention also concerns preparations of cells (e.g., hepatocytes) that have been repeatedly cryopreserved and thawed.

11 Claims, No Drawings

OTHER PUBLICATIONS

Lawrence, J.N. et al. (1991) "*Development Of An Optimal Method For The Cryopreservation Of Hepatocytes And Their Subsequent Monolayer Culture. Toxicology* In Vitro," 5(1):39-51.

Lee, S. W. et al. (2004) "*Hepatocyte Transplantation: State Of The Art And Strategies For Overcoming Existing Hurdles," Ann. Hepatol.* 3(2):48-53.

Lemaigre, F. et al. (2004) "*Liver Development Update: New Embryo Models, Cell Lineage Control, And Morphogenesis*," Curr Opin Genet Dev. 14(5):582-590.

Li, A.P. et al. (1992) "*Isolation And Culturing Of Hepatocytes From Human Liver*," J. Tissue Cult. Meth. 14:139-146.

Li, A.P. et al. (1999) "*Cryopreserved Human Hepatocytes: Characterization Of Drug-Metabolizing Enzyme Activities And Applications In Higher Throughput Screening Assays For Hepatotoxicity, Metabolic Stability, And Drug-Drug Interaction Potential*," Chem Biol Interact. 121(1):17-35.

Li, A.P. et al. (1999) "*Present Status Of The Application Of Cryopreserved Hepatocytes In The Evaluation Of Xenobiotics: Consensus Of An* International *Expert Panel*," Chem Biol Interact. 121(I):117-123.

Lloyd, T.D.R. et al. (2003) *Cryopreservation Of Hepatocytes: A Review Of Current Methods For Banking, Cell and Tissue Culture Banking* 4:3-15.

Loretz, L.J. et al. (1989) "*Optimization Of Cryopreservation Procedures For Rat And Human Hepatocytes*," Xenobiotica. 19(5):489-498.

Madan, A. et al. (1999)"*Effect of Cryopreservation on Cytochrome P-450 Enzyme Induction in Cultured Rat Hepatocytes*," Drug Metab. Dispos. 27(3):327-335.

Meng, Q. et al. (2004) "*Hepatocyte Culture In Bioartificial Livers With Different Membrane Characteristics*," Biotechnol Lett. 26(18):1407-1412.

Morsiani et al., (1995) "*Automated Liver Cell Processing Facilitates Large Scale Isolation And Purification Of Porcine Hepatocytes," ASAIO Journal* 41:155-161.

Nanji, A.A. (2004) "*Animal Models Of Nonalcoholic Fatty Liver Disease And Steatohepatitis*," Clin Liver Dis. 8(3):559-574.

Novicki, D.L. et al. (1982) "*Cryopreservation Of Isolated Rat Hepatocytes*," In Vitro. 18(4):393-399.

O'Brien, Z.Z. et al. (undated) "*The Construction Of A Representative Human Cryopreserved Hepatocyte Pool For Metabolism Study*,".

Ponsoda, X. et al. (2004) "*Drug Metabolism By Cultured Human Hepatocytes: How Far Are We From The* In Vivo *Reality?*" Altern Lab Anim. 32(2): 101-110.

Postic, C. et al. (2004) "*Role Of The Liver In The Control Of Carbohydrate And Lipid Homeostasis*," Diabetes Metab. 30(5):398-408.

Roymans, D. et al. (2004) "*Determination Of Cytochrome P450 1A2 And Cytochrome P4503a4 Induction In Cryopreserved Human Hepatocytes*," Biochem Pharmacol. 67(3):427-437.

Ruegg, C.E. et al. (1997) "*Cytochrome-P450 Induction and Conjugated Metabolism In Primary Human Hepastocytes After Cryopreservation*," In Vitro Toxicol. 10:217-222.

Seglen, P.O. (1976) "*Preparation Of Isolated Rat Liver Cells*," Meth. Cell Biol. 13:29-83).

Sekido, H. el al. (2004) "*Usefulness Of Artificial Liver Support For Pretransplant Patients With Fulminant Hepatic Failure*," Transplant Proc. 36(8):2355-2356.

Shaddock, J,G. et al. (1993) "*Cryopreservation And Long-Term Storage Of Primary Rat Hepatocytes: Effects On Substrate-Specific Cytochrome P450-Dependent Activities And Unscheduled DNA Synthesis*," Cell Biol Toxicol. 9(4):345-357.

Silva, J.M. et al. (1999) "*Induction Of Cytochrome-P450 In Cryopreserved Rat And Human Hepatocytes*," Chem-Biol Interact 121:49-63.

Sun, E.L. et al. (1990) "*Cryopreservation Of Cynomologus Monkey (Macaca fascicularis) Hepatocytes For Subsequent Culture And Protein Synthesis Studies,"* In vitro *Cell Development and Biology* 25:147-150.

Utesch, D. et al. (1992) "*Characterization Of Cryopreserved Rat Liver Parenchymal Cells By Metabolism Of Diagnostic Substrates And Activities Of Related Enzymes," Biochemical Pharmacology* 44:309-315.

Zaleski, J. et al. (1993) "*Preservation Of The Rate And Profile Of Xenobiotic Metabolism In Rat Hepatocytes Stored In Liquid Nitrogen*," Biochem Pharmacol. 46(1):111-116.

Zhang, J.G. et al. (undated) "Validation of Pooled Cryopreserved Human Hepatocytes as a Model for Metabolism Studies" www.Bdbiosciences.com.

Li et al., Present status of the application of cryopreserved hepatocytes in the evaluation of xenobiotics . . . , 121 Chemico-Biological Interactions 117-123 (1999).

Li, Overview: hepatocytes and cryopreservation—a personal historical perspective, 121 Chemico-Biological Interactions 1-5 (1999).

* cited by examiner

CELLULAR COMPOSITIONS AND METHODS FOR THEIR PREPARATION

FIELD OF THE INVENTION

The present invention relates to novel cell (e.g., hepatocyte, etc.) compositions and methods for their preparation and use. In particular, the invention concerns methods of processing preparations of such cells so as to permit their repeated cryopreservation and thawing while retaining substantial viability. The invention also concerns preparations of cells (e.g., hepatocytes) that have been repeatedly cryopreserved and thawed.

BACKGROUND OF THE INVENTION

Hepatocytes are parenchymal liver cells, and make up 60-80% of the cytoplasmic mass of the liver. Hepatocytes play a key role in the detoxification, modification and excretion of exogenous and endogenous substances (Ponsoda, X. et al. (2004) "*Drug Metabolism By Cultured Human Hepatocytes: How Far Are We From The In Vivo Reality?*" Altern Lab Anim. 32(2): 101-110). One of the detoxifying functions of hepatocytes is to modify ammonia to urea for excretion. They are also important in protein synthesis and storage, in the transformation of carbohydrates and in the synthesis of cholesterol, bile salts and phospholipids (Postic, C. et al. (2004) "*Role Of The Liver In The Control Of Carbohydrate And Lipid Homeostasis,*" Diabetes Metab. 30(5):398-408). The hepatocyte is the only cell in the body that manufactures albumin, fibrinogen, and the prothrombin group of clotting factors. It is the main site for the synthesis of lipoproteins, ceruloplasmin, transferrin, and glycoproteins. Hepatocytes manufactures their own structural proteins and intracellular enzymes. Hepatocytes are also important depots for vitamin B12 and iron.

Due to these attributes, isolated and cultured hepatocytes have become very attractive as models systems for the study of liver functions (Chesne, C. et al. (1993) "*Viability And Function In Primary Culture Of Adult Hepatocytes From Various Animal Species And Human Beings After Cryopreservation,*" Hepatology 18(2):406-414; Guillouzo, A. et al. (1986) "*Isolated and Cultured Hepatocytes,*" Paris: les Editions INSERM and London: John Libbey Eurotext); Ponsoda, X. et al. (2004) "*Drug Metabolism By Cultured Human Hepatocytes: How Far Are We From The In Vivo Reality?*" Altern Lab Anim. 32(2): 101-110; Gomez-Lechon, M. J. et. al. (2004) "*Human Hepatocytes In Primary Culture: The Choice To Investigate Drug Metabolism In Man,*" Curr Drug Metab. 5(5):443-462; Lemaigre, F. et al. (2004) "*Liver Development Update: New Embryo Models, Cell Lineage Control, And Morphogenesis,*" Curr Opin Genet Dev. 14(5):582-590; Nanji, A. A. (2004) "*Animal Models Of Nonalcoholic Fatty Liver Disease And Steatohepatitis,*" Clin Liver Dis. 8(3):559-574; Hewitt, N. J. et al. (2004) *Cryopreserved Rat, Dog And Monkey Hepatocytes: Measurement Of Drug Metabolizing Enzymes In Suspensions And Cultures,*" Hum Exp Toxicol. 23(6):307-316).

In addition to their use in liver models, hepatocytes have the potential of being used to produce Bioartificial Livers (BALs) or in hepatocyte transplantation that can provide liver functions for individuals suffering from liver disease or liver failure. Bioartificial Livers (BALs) are described by Anand, A. C. (1996) *"Bioartificial Livers: The State Of The Art,"* Trop Gastroenterol. 17(4):197-198, 202-211; Gan, J. H. et al. (2005) "*Hybrid Artificial Liver Support System For Treatment Of Severe Liver Failure,*" World J Gastroenterol. 11 (6):890-894; Fukuda, J. et al. (2004) "*Hepatocyte Organoid Culture In Elliptic Hollow Fibers To Develop A Hybrid Artificial Liver,*" Int J Artif Organs. 27(12): 1091-1099; Meng, Q. et al. (2004) "*Hepatocyte Culture In Bioartificial Livers With Different Membrane Characteristics,*" Biotechnol Lett. 26(18): 1407-1412; Sekido, H. et al. (2004) "*Usefulness Of Artificial Liver Support For Pretransplant Patients With Fulminant Hepatic Failure,*" Transplant Proc. 36(8):2355-2356; WO03/105663A2, WO05/000376A2, and U.S. Pat. No. 6,759,245. Hepatocyte transplantation is described by Chan, C. et al. (2004) "*Hepatic Tissue Engineering For Adjunct And Temporary Liver Support: Critical Technologies,*" Liver Transpl. 10(11): 1331-1342; Lee, S. W. et al. (2004) "*Hepatocyte Transplantation: State Of The Art And Strategies For Overcoming Existing Hurdles,*" Ann. Hepatol. 3(2):48-53; Horslen, S. P. (2004) "*Hepatocyte Transplantation,*" Transplantation 77(10):1481-1486; Burlina, A. B. (2004) "*Hepatocyte Transplantation For Inborn Errors Of Metabolism,*" J. Inherit. Metab. Dis. 27(3):373-83; and Fox, I. J. et al. (2004) "*Hepatocyte Transplantation,*" Am. J. Transplant. 4 Suppl. 6:7-13.

A limiting factor in the development of such model systems and to the development of Bioartificial Livers (BALs) has been the erratic source and limited availability of hepatocytes, especially human hepatocytes. Fresh hepatocytes are obtainable only from liver resections or non-transplantable livers of multi-organ donors (Lloyd, T. D. R. et al. (2003) *Cryopreservation Of Hepatocytes: A Review Of Current Methods For Banking," Cell and Tissue Culture Banking* 4:3-15). The supply of such tissue is inconsistent and often geographically inconvenient in light of the limited functional lifespan of liver tissue (Smrzova, J. et al. (2001) "*Optimization Of Porcine Hepatocytes Cryopreservation By Comparison Of Viability And Enzymatic Activity Of Fresh And Cryopreserved Cells,*" Acta Veterinaria Brunensis 70:141-147).

One approach to addressing this problem has involved the development of hepatocyte storage conditions that allow hepatocytes to be maintained over time with their cellular functions preserved. Cryopreservation methods for the storage of hepatocytes have been developed to address this need (see, Lloyd, T. D. R. et al. (2003) *Cryopreservation Of Hepatocytes: A Review Of Current Methods For Banking," Cell and Tissue Culture Banking* 4:3-15; Loretz, L. J. et al. (1989) "*Optimization Of Cryopreservation Procedures For Rat And Human Hepatocytes,*" Xenobiotica. 19(5):489-498; Shaddock, J, G. et al. (1993) "*Cryopreservation And Long-Term Storage Of Primary Rat Hepatocytes: Effects On Substrate-Specific Cytochrome P450-Dependent Activities And Unscheduled DNA Synthesis,*" Cell Biol Toxicol. 9(4):345-357; Novicki, D. L. et al. (1982) "*Cryopreservation Of Isolated Rat Hepatocytes,*" In Vitro. 18(4):393-399; Zaleski, J. et al. (1993) "*Preservation Of The Rate And Profile Of Xenobiotic Metabolism In Rat Hepatocytes Stored In Liquid Nitrogen ,*" Biochem Pharmacol. 46(1):111-116). Typically, such measures comprise storage in liquid nitrogen (−196° C.) or in frozen nitrogen gas (−150° C.). The ability to recover viable thawed cells has been found to depend on multiple factors such as the rate of freezing, the concentration of hepatocytes, the type of cryoprotectant employed, and the final cooling temperature. Cell concentrations of 10^6-10^7 cells/ml have been typically employed. The isolated hepatocytes are typically incubated in suspension for a period (e.g., 4-48 hours) to allow them to recover from the isolation process. Thereafter, a cryoprotectant (such as glycerol, DMSO, polyvinylpyrrolodine, or dextran) is added, and the hepatocytes are frozen. The art has developed various freezing procedures, all designed to minimize or prevent the occurrence of intracellular ice. The

freezing rates typically vary from −0.05° C./min to −50° C./min (see, Lloyd, T. D. R. et al. (2003) *Cryopreservation Of Hepatocytes: A Review Of Current Methods For Banking," Cell and Tissue Culture Banking* 4:3-15).

While the development of cryopreservation methods for the storage of hepatocytes has significantly facilitated the availability of human hepatocytes, cryopreservation has been found to cause a significant decrease in cellular viability (e.g., 25-35%) (Dou, M. et al. (1992) "*Thawed Human Hepatocytes In Primary Culture,*" Cryobiology 29:454-469; Alexandre, E. et al. (2002) "*Cryopreservation Of Adult Human Hepatocytes Obtained From Resected Liver Biopsies,*" Cryobiology 44:103-113). Coundouris, J. A. et al. (1993) reported viability of 67% after 24 hours, declining to 49% after 14 days (Coundouris, J.A. et al. (1993) "*Cryopreservation Of Human Adult Hepatocytes For Use In Drug Metabolism And Toxicity Studies,*" Xenobiotica. 23(12):1399-1409). Adams, R. M. et al. have reported that the viability of hepatocytes may be enhanced to greater than 90% using specialized cyropreservation fluids, however, only 16% of cells were found to be capable of replication (Adams, R. M. et al. (1995) "*Effective Cryopreservation And Long-Term Storage Of Primary Human Hepatocytes With Recovery Of Viability, Differentiation, And Replicative Potential,*" Cell Transplant. 4(6):579-586). Methods of cryopreservation are disclosed in U.S. Pat. Nos. 5,795,711, 6,136,525, 5,895,745; International Patent Publications WO04/009766, WO92/12722, WO/0153462, European Patent No. EP0834252B, and U.S. Patent Application Publication Nos. US20020039786A1, US20030134418A1. The poor recovery of cells when cryopreserved continues to limit the use of hepatocytes in in vitro liver models.

A second major problem affecting the use of both fresh and cryopreserved hepatocytes is the variation of liver enzyme expression that is observed in tissue from different donors (Li, A. P. et al. (1999) "*Present Status Of The Application Of Cryopreserved Hepatocytes In The Evaluation Of Xenobiotics: Consensus Of An International Expert Panel,*" Chem Biol Interact. 121(1):117-123; Li, A. P. et al. (1999) "*Cryopreserved Human Hepatocytes: Characterization Of Drug-Metabolizing Enzyme Activities And Applications In Higher Throughput Screening Assays For Hepatotoxicity, Metabolic Stability And Drug-Drug Interaction Potential,*" Chem Biol Interact. 121(1):17-35; O'Brien, Z. Z. et al. (undated) "*The Construction Of A Representative Human Cryopreserved Hepatocyte Pool For Metabolism Study.*" One solution to this sample variation involves pooling samples from different sources to produce a "composite" hepatocyte preparation having the characteristics of "average" liver cells. However, the frequency of receipt of fresh tissue and the need to cryopreserve hepatocytes immediately after isolation has been cited as preventing the preparation of hepatocyte pools. Thus, multiple companies (e.g., Xenotech, LLC; BD Biosciences) refrain from selling pooled hepatocytes thus forcing the end user to thaw and pool hepatocytes from several different donors. This difficulty remains even though pooled cryopreserved human hepatocytes are a valid model for metabolic studies (Zhang, J. G. et al. (undated) "*Validation Of Pooled Cryopreserved Human Hepatocytes As A Model For Metabolic Studies.*"

Thus, despite all prior advances, a need remains for processes that would enable the availability of hepatocytes for medical research and other purposes. A need further exists for a stable and reproducible source of human hepatocytes. The present invention permits the production and availability of hepatocyte preparations that may be repeatedly cryopreserved and thawed without unacceptable loss of viability. The invention thus permits multiple hepatocyte samples to be pooled to produce pooled hepatocyte preparations, especially pooled cryopreserved human hepatocyte preparations. Using such advance, pooled cryopreserved human hepatocytes are now commercially available from In Vitro Technologies (Baltimore, Md.).

SUMMARY OF THE INVENTION

The present invention relates to novel cell (e.g., hepatocyte) compositions and methods for their preparation and use. In particular, the invention concerns methods of processing preparations of cells, especially hepatocytes, so as to permit their repeated cryopreservation and thawing while retaining substantial viability. The invention also concerns preparations of cells (e.g., hepatocytes) that have been repeatedly cryopreserved and thawed.

In detail, the invention particularly concerns a multi-cryopreserved hepatocyte preparation comprising hepatocytes that have been frozen and thawed at least two times, wherein greater than 50% and more preferably 70% or more of the hepatocytes of the preparation are viable.

The invention further concerns the embodiment of such a multi-cryopreserved hepatocyte preparation wherein the hepatocytes are selected from the group consisting of human hepatocytes, porcine hepatocytes, simian hepatocytes, canine hepatocytes, feline hepatocytes, bovine hepatocytes, equine hepatocytes, ovine hepatocytes and rodent hepatocytes.

The invention further concerns the embodiment of such a multi-cryopreserved hepatocyte preparation wherein the preparation comprises a pooled preparation of hepatocytes of multiple sources, which may be of the same or different gender, race, or health state, or which provide the pooled preparation with a desired level of a metabolic activity (especially wherein the metabolic activity is selected from the group consisting of COUM, DEX, ECOD, 7-HCG, 7-HCS, MEPH, TEST, PHEN and CZX).

The invention further concerns a method of producing a desired preparation of multi-cryopreserved hepatocytes, the hepatocytes being capable of being frozen and thawed at least two times, and in which greater than 50% and more preferably 70% or more of the hepatocytes of the preparation are viable, the method comprising:

(A) subjecting hepatocytes that have been frozen and thawed to density gradient fractionation (especially percoll density centrifugation) to separate viable hepatocytes from non-viable hepatocytes,

(B) recovering the separated viable hepatocytes, and

(C) cryopreserving the recovered viable hepatocytes to thereby form the desired preparation of hepatocytes.

The invention further concerns the embodiment of such a method in which the hepatocytes are selected from the group consisting of human hepatocytes, porcine hepatocytes, simian hepatocytes, canine hepatocytes, feline hepatocytes, bovine hepatocytes, equine hepatocytes, ovine hepatocytes and rodent hepatocytes.

The invention further concerns the embodiment of such a method in which the preparation comprises a pooled preparation of hepatocytes of multiple sources, which may be of the same or different gender, race, or health state, or which provide the pooled preparation with a desired level of a metabolic activity (especially wherein the metabolic activity is selected from the group consisting of COUM, DEX, ECOD, 7-HCG, 7-HCS, MEPH, TEST, PHEN and CZX).

The invention also concerns a method of investigating in vitro drug metabolism comprising incubating hepatocytes of a multi-cryopreserved hepatocyte preparation in the presence

of a xenobiotic, and determining the metabolic fate of the xenobiotic, or the effect of the xenobiotic on the hepatocytes or on an enzyme or metabolic activity thereof, wherein the hepatocytes have been frozen and thawed at least two times, and wherein greater than 50% and more preferably 70% or more of the hepatocytes of the preparation are viable.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to novel cell compositions and methods for their preparation and use. In particular, the invention concerns methods of processing preparations of cells so as to permit their repeated cryopreservation and thawing while retaining substantial viability. The methods of the present invention are generally applicable to a wide variety of cell types, including hepatocytes, kidney cells, spleen cells, thymus cells, bone marrow cells, stem cells, muscle cells (including cardiac muscle cells), endocrine cells (including pancreatic cells, adrenal cells, thyroid cells, etc.) epidermal cells, endodermal cells, etc. The methods of the present invention are illustrated below with respect to a preferred cell type: hepatocytes.

The invention also concerns preparations of cells, e.g., hepatocytes, that have been repeatedly cryopreserved and thawed to obtain a high viability preparation useful for a variety of experimental, diagnostic and therapeutic purposes. The present invention extends the ability of hepatocytes to be cryopreserved and thawed for later use, so as to permit hepatocyte preparations to be repeatedly cryopreserved and thawed without an unacceptable loss of viability.

As used herein, the term "cell preparation" denotes a liquid or frozen composition of cells from one or more sources (e.g., "hepatocyte preparation" denotes a composition of liver cells from one or more sources). The sources may be primary cells that have been dissociated from or isolated from tissue as by resection, biopsy, or from donor organs, or they may be secondary, immortalized or transformed cell cultures. The cells may be derived from any mammalian source, including human, porcine, simian, canine, feline, bovine, equine, ovine or rodent sources. The use of human, porcine or rodent (especially rat) cells is preferred. Preferably, greater than 50% and more preferably 70% or more of the hepatocytes of such preparations will be viable.

As used herein, the term "multi-cryopreserved cell preparation" denotes a cell preparation that has been frozen and then thawed at least two times (e.g., a "multi-cryopreserved hepatocyte preparation" denotes a hepatocyte preparation that has been frozen and then thawed at least two times). Such preparations may have been frozen and thawed three, four, five, or more times.

The term "pooled preparation" denotes a cell (e.g., hepatocyte) preparation in which the cells (e.g., hepatocytes) are derived from two, three, four, five, or more different sources, such as different donors, biopsies, tissue resections from different tissue samples or different tissue sources, or different primary, secondary, immortalized or transformed cell (e.g., hepatocyte) cultures. The cells of such pooled preparations may be randomly selected cell, or may have been selected to provide the pooled preparation with a desired level of one or more metabolic activities (such as for example, a preparation of hepatocytes having a desired level of COUM, DEX, ECOD, 7-HCG, 7-HCS, MEPH, TEST, PHEN and/or CZX activity), or a desired cell characteristic (such as, for example, a preparation of hepatocytes derived from sources of the same gender, age, race (e.g., Caucasian, etc.), or health state (e.g., hepatocytes of hepatitis virus-infected liver, hepatocytes of HIV-1 infected liver, hepatocytes of healthy liver, hepatocytes of cigarette smokers, hepatocytes of individuals suffering from cirrhosis of the liver, or from other diseases or conditions). For example, to obtain a pooled hepatocyte preparation with minimal DEX activity, a pooled preparation could be prepared from Lot. Nos. 067, CEK, ETR, PFM, VTA, or WWM (see, Table III).

In a preferred embodiment, illustrated with respect to hepatocyte cells, the practice of the invention comprises some or all of the following steps: the isolation of hepatocytes, a first cryopreservation of the isolated primary hepatocytes to obtain a first cryopreserved hepatocyte preparation, the thawing of the first cryopreserved hepatocyte preparation to obtain viable hepatocytes, and the reformulation of the thawed viable hepatocytes to permit their further storage and use through repeated cryopreservation and thawing to obtain viable hepatocytes.

The Isolation of Hepatocytes

Any of a wide variety of methods may be employed or adapted to permit the isolation of the primary hepatocytes used in the present invention. For example, suitable techniques for the isolation of hepatocytes are outlined in Morsiani et al., (1995) "*Automated Liver Cell Processing Facilitates Large Scale Isolation And Purification Of Porcine Hepatocytes,*" *ASAIO Journal* 41:155-161 and in Seglen, P. O. (1976) "*Preparation Of Isolated Rat Liver Cells,*" Meth. Cell Biol. 13:29-83). Specific reference is made to the two-step collagenase digestion procedure described in Li, A. P. et al. (1992) "*Isolation And Culturing Of Hepatocytes From Human Liver,*" J. Tissue Cult. Meth. 14:139-146.

The hepatocytes may be cultured in any suitable hepatocyte culture medium. By way of illustration and not limitation mention may be made of the following culture media Chee's Essential Media (Hamilton, G. A. et al. (2001) "*Effects Of Medium Composition On The Morphology And Function Of Rat Hepatocytes Cultured As Spheroids And Monolayers,*" In Vitro Cell Dev Biol Anim. 37(10):656-667; Zurlo, J. et al. (1996) "*Characterization Of A Primary Hepatocyte Culture System For Toxicological Studies,*" In Vitro Cell Dev Biol Anim. 32(4):211-220; Arterburn, L. M. et al. (1995) "*A Morphological Study Of Differentiated Hepatocytes In Vitro,*" Hepatology 22(1):175-187), Modified Eagle Medium (or Dulbecco's Modified Eagle Medium) (Arikura, J. et al. (2002) "*UW Solution: A Promising Tool For Cryopreservation Of Primarily Isolated Rat Hepatocytes,*" J Hepatobiliary Pancreat Surg. 9(6):742-749; Washizu, J. et al. (2000) "*Amino Acid Supplementation Improves Cell-Specific Functions Of The Rat Hepatocytes Exposed To Human Plasma,*" Tissue Eng. 6(5):497-504; Iwata, H. et al. (1999) "*In Vitro Evaluation Of Metabolic Functions Of A Bioartificial Liver,*" ASAIO J. 45(4):299-306; Stutenkemper, R. et al. (1992) "*The Hepatocyte-Specific Phenotype Of Murine Liver Cells Correlates With High Expression Of Connexin*32 *And Connexin*26 *But Very Low Expression Of Connexin*43," Exp Cell Res. 201(1): 43-54), Leibowitz medium (Coundouris, J. A. et al. (1993) "*Cryopreservation Of Human Adult Hepatocytes For Use In Drug Metabolism And Toxicity Studies,*" Xenobiotica. 23(12):1399-1409), Waymouth (Vind, C. et al. (1992) "*Regulation By Growth Hormone And Glucocorticoid Of Testosterone Metabolism In Long-Term Cultures Of Hepatocytes From Male And Female Rats,*" Biochem Pharmacol. 44(8):1523-1528; Nemoto, N. et al. (1991) "*Proline Is Required For Transcriptional Control Of The Aromatic Hydrocarbon-Inducible P*(1)450 *Gene In C57BL/6 Mouse Monolayer-Cultured Hepatocytes,*" Jpn J Cancer Res. 82(8):901-908; Dich, J. et al. (1988) "*Long-Term Culture Of Hepatocytes: Effect Of*

Hormones On Enzyme Activities And Metabolic Capacity," Hepatology. 8(1):39-45; Goethals, F. et al. (1984) "*Critical Biochemical Functions Of Isolated Hepatocytes As Sensitive Indicators Of Chemical Toxicity,"* Fundam Appl Toxicol. 4(3 Pt 1):441-450), Kreb's medium (House, J. D. (2001) "*Threonine Metabolism In Isolated Rat Hepatocytes,"* Am J Physiol Endocrinol Metab. 281(6):E1300-1307; Irvine, F. et al. (1993) "*Extracellular Calcium Modulates Insulin's Action On Enzymes Controlling Cyclic AMP Metabolism In Intact Hepatocytes,"* Biochem J. 293 (Pt 1):249-253; Marsh, D. C. et al. (1991) "*Hypothermic Preservation Of Hepatocytes. III. Effects Of Resuspension Media On Viability After Up To 7 Days Of Storage,"* Hepatology 13(3):500-508), etc.

In a preferred embodiment, hepatocytes are cryopreserved in a medium containing approximately 10% DMSO and approximately 90% fetal bovine serum (Loretz, L. J. et al. (1989) "*Optimization Of Cryopreservation Procedures For Rat And Human* Hepatocytes," *Xenobiotica* 19:489-498; Ruegg, C. E. et al. (1997) "*Cytochrome-P450 Induction and Conjugated Metabolism In Primary Human Hepastocytes After Cryopresevation,"* In Vitro Toxicol. 10:217-222).

The viability of the isolated hepatocytes may be determined using any of a variety or methods. Preferable, such viability will be determined using the Trypan blue exclusion method (see, e.g., Berry, M. N. et al. (1992) "*Techniques For Pharmacological And Toxicological Studies With Isolated Hepatocyte Suspensions,"* Life Sci. 51(1):1-16). Thus the phrases "viable hepatocytes" or "percent viability", as used herein, refers to hepatocyte viability as assessed using the method of Trypan Blue exclusion.

Cryopreservation of the Isolated Primary Hepatocytes

The hepatocytes of the present invention are preferably cryopreserved using liquid nitrogen, and most preferably within 36 hours of their isolation. Considerations for the cryopreservation of human hepatocytes are discussed in Lloyd, T. D. R. et al. (2003) *Cryopreservation Of Hepatocytes: A Review Of Current Methods For Banking," Cell and Tissue Culture Banking* 4:3-15. Suitable procedures for the cryopreservation of hepatocytes may also be found in the following documents: Adams, R. M. et al. (1995) "*Effective Cryopreservation And Long-Term Storage Of Primary Human Hepatocytes With Recovery Of Viability, Differentiation, And Replicative Potential,"* Cell Transplant. 4(6):579-586; Chesne, C. et al. (1993) "*Viability And Function In Primary Culture Of Adult Hepatocytes From Various Animal Species And Human Beings After Cryopreservation,"* Hepatology 18(2):406-414; Coundouris, J. A. et al. (1993) "*Cryopreservation Of Human Adult Hepatocytes For Use In Drug Metabolism And Toxicity Studies," Xenobiotica.* 23(12): 1399-1409; Hewitt, N. J. et al. (2004) *Cryopreserved Rat, Dog And Monkey Hepatocytes: Measurement Of Drug Metabolizing Enzymes In Suspensions And Cultures," Hum Exp Toxicol.* 23(6):307-316; Novicki, D. L. et al. (1982) "*Cryopreservation Of Isolated Rat Hepatocytes,"* In Vitro. 18(4):393-399; Shaddock, J, G. et al. (1993) "*Cryopreservation And Long-Term Storage Of Primary Rat Hepatocytes: Effects On Substrate-Specific Cytochrome P450-Dependent Activities And Unscheduled DNA Synthesis,"* Cell Biol Toxicol. 9(4):345-357; Zaleski, J. et al. (1993) "*Preservation Of The Rate And Profile Of Xenobiotic Metabolism In Rat Hepatocytes Stored In Liquid Nitrogen,"* Biochem Pharmacol. 46(1):111-116.

Preferably, isolated hepatocytes are suspended in a cryoprotective medium, and the suspended cells are dispensed into freezer-safe containers. A cryoprotective medium typically comprises a hepatocyte culture medium that contains at least one cryoprotectant that minimizes the deleterious effects of cryopreservation such as the formation of intracellular ice during freezing. By way of illustration and not limitation, the following commonly used cryoprotectants are listed: dimethylsulfoxide (DMSO), polyethylene glycol, amino acids, propanediol, and glycerol. A preferred cryoprotectant of the present invention is DMSO. Suitable cryoprotectants and methods for their use in hepatocyte cryopreservation can be found, for example, in: Loretz, L. J. et al. (1989) "*Optimization Of Cryopreservation Procedures For Rat And Human Hepatocytes,"* Xenobiotica. 19(5):489-498; Chesne, C. et al. (1993) "*Viability And Function In Primary Culture Of Adult Hepatocytes From Various Animal Species And Human Beings After Cryopreservation,"* Hepatology 18(2):406-414; Diener, B. et al. (1993) "*A Method For The Cryopreservation Of Liver Parenchymal Cells For Studies Of Xenobiotics,"* Cryobiology 30(2):116-127; Lawrence, J. N. et al. (1991) "*Development Of An Optimal Method For The Cryopreservation Of Hepatocytes And Their Subsequent Monolayer Culture. Toxicology In Vitro,"* 5(1):39-51; Houle, r. et al. (2003) "*Retention of Transporter Activities in Cryopreserved. Isolated Rat Hepatocytes,"* Drug Metab. Disposit. 31(4):447-451; Silva, J. M. et al. (1999) "*Induction Of Cytochrome-P450 In Cryopreserved Rat And Human Hepatocytes,"* Chem-Biol Interact 121:49-63.

The isolated hepatocytes are preferably suspended in a cryoprotective medium in preparation for freezing. The suspended cells are preferably dispensed into freezer resistant containers at a cell density of from about 10^6 cells/ml to about 4×10^7 cells/ml. Preferred freezing volumes range from 0.1-10.0 ml. The preferred freezing volume is 1.0 ml.

The dispensed hepatocytes are then preferably cryopreserved using a controlled rate freezing process, most preferably at a freezing rate of between about −1° C./min to about −25° C./min until a final temperature of about −90° C. is reached. During the initial phase of the cryopreservation process, seeding may be employed to induce controlled crystallization or ice formation in cell suspensions that have already been cooled to below the freezing point of the culture medium. Such seeding serves to minimize ice formation-related damage and therefore may be beneficial to cell viability. Suitable seeding methods include inserting a cold metal rod into the freezing containers, and introducing a blast of liquid nitrogen into the freezing containers.

Once the desired final temperature has been reached, the frozen cell samples may be transferred to liquid nitrogen freezers for prolonged storage. The frozen samples may be stored in either the liquid nitrogen phase or the gas phase of liquid nitrogen. Preferably storage is accomplished in the gas phase of liquid nitrogen. The frozen samples may be stored in this manner for days, months, or years, with the length of storage in the gas phase of liquid nitrogen having little effect on the post-thaw viability and function.

The Thawing of Cryopreserved Hepatocytes

Frozen samples may be thawed for further processing by removing them from the presence of liquid nitrogen or liquid nitrogen vapor. Frozen samples are preferably thawed by placing the samples immediately into a prewarmed water bath having a temperature of between about 37° C. to about 42° C. Preferably, cells are thawed to at least the stage in which ice chunks can be dislodged when the sample container is inverted. The thawed cells are then preferably rapidly processed to remove the cells from contact with DMSO, for example by PERCOLL® (colloidal silica particles of 15-30 nm diameter (23% w/w in water) which have been coated

with polyvinylpyrrolidone (PVP)) gradient centrifugation (as described below) or by sequential washings.

In a preferred embodiment, the cells are thawed into Complete INVITROGRO™ CP medium (In Vitro Technologies, Baltimore, Md.; Roymans, D. et al. (2004) "*Determination Of Cytochrome P*450 *IA2 And Cytochrome P*4503*a*4 *Induction In Cryopreserved Human Hepatocytes,*" Biochem Pharmacol. 67(3):427-437) (hepatocyte plating medium, which contains water, Dulbecco's Modified Eagle Medium, sodium bicarbonate, HEPES, fructose, bovine serum albumin, sodium hydroxide, MEM non-essential amino acids, insulin, hydrocortisone, and newborn calf serum). The medium is prepared by thawing TORPEDO™ Antibiotic Mix (In Vitro Technologies, Baltimore, Md.) (a mixture of antibiotics selected to inhibit bacterial growth in hepatocyte cell cultures that contains penicillin, streptomycin, gentamicin, amikacin and fungizone) to 37° C. in a water bath until thawed, and then removed from the water bath. 1.0 ml of TORPEDO™ Antibiotic Mix is then mixed with 45 ml INVITROGRO™ CP medium. Following the addition of TORPEDO™ Antibiotic Mix, the shelf life for the complete medium is 7 days. When thawing a single vial, the INVITROGRO™ CP medium is prewarmed to approximately 37° C. 5 ml of warmed INVITROGRO™ CP medium is added to a sterile 50 ml conical tube. The vial of frozen hepatocytes is carefully removed from the freezer. If the vial was stored in the liquid phase, its cap is carefully removed, any liquid nitrogen present in the vial is decanted, and the cap is reclosed before placing the vial into the water bath. It is preferred to then immediately immerse the vial into a 37° C. water bath, and to shake the vial gently until the ice is entirely melted, but no longer than it takes to completely thaw the vial. It may be helpful to remove any labels from the vial so that it will be easier to view the vial contents. The thawed contents are then emptied into the prewarmed INVITROGRO™ CP medium. 1.0 ml of prewarmed INVITROGRO™ CP medium then is added to each vial to resuspend any remaining cells. The contents of the vial are then decanted or pipetted into the hepatocyte suspension. The hepatocytes are preferably resuspended by gently inverting the receiving container (e.g., vial, test tube, etc.) several (e.g., three) times.

When thawing multiple vials, it is preferred that all of the vials be thawed in the water bath simultaneously. As before, the medium (preferably, INVITROGRO™ CP medium) should be warmed to 37° C. It is desirable to ensure that there is enough medium to permit 5 ml of pre-warmed INVITROGRO™ CP medium to be used for each vial of cryopreserved hepatocytes. After vials have thawed, their caps should be quickly removed and their contents poured into a sterile tube or beaker that contains at least 5 ml of pre-warmed INVITROGRO™ CP medium per vial thawed. For example, 25 ml of media is preferably employed for 5 vials in a container that can hold a volume of 50 ml.

If desired, the total cell count and the number of viable cells may be determined using the Trypan Blue exclusion method. Cells may be diluted to 0.70×10^6 viable cells/ml with INVITROGRO™ CP medium.

The Reformulation of Thawed Hepatocytes to Permit Further Cryopreservation and Thawing

One aspect of the present invention concerns the ability to reformulate the thawed cells so that they may be refrozen and rethawed on one or more subsequent occasions. Such multi-cryopreserved hepatocyte preparations have multiple uses. They may be used in bioartificial livers, liver cell transplants, liver assist devices, hepatocyte transplantations, and in vitro applications. In particular, multi-cryopreserved hepatocyte preparations may be used in in vitro drug metabolism studies (for example, in identifying hepatocytes with unique characteristics (e.g.,. metabolic polymorphisms, genetic polymorphisms, etc.), in studies on the metabolic fate of the xenobiotic and studies on the affect of the xenobiotic in altering the drug-metabolizing enzyme profile of the hepatocytes, in inhibition studies to determine the IC_{50} of xenobiotics on liver enzymes and functions (e.g. cholesterol metabolism), in gene induction studies with xenobiotics, in protein induction studies with xenobiotics, in toxicity assessment of xenobiotics on hepatocytes, transport studies with xenobiotics (e.g. studies on P-glycoprotein transport systems, organic ion transporters, organic cation transporters, etc.), in metabolic clearance studies with xenobiotics, and in efficacy assays (e.g. lipoprotein processing, gluconeogenesis, protein secretion etc.). Multi-cryopreserved hepatocyte preparations may also be used to study or propagate hepatitis viruses and other infectious viruses and agents. Recovered cells may be reformulated for use in DNA, mRNA or proteomic studies or in studies of metabolic polymorphisms. Multi-cryopreserved hepatocyte preparations may also be used in metabolic clearance studies and efficacy assays (e.g., lipoprotein processing, gluconeogenesis, protein secretion, etc.). Cells may be reformulated for use in seeding bioreactors for large scale incubations or as models for gene regulation via micro RNA, or for use in combination systems with other cell types (e.g. non-parenchymal cells from liver or cells from other sources, e.g. Caco-2 cells).

In a preferred embodiment of the invention, such reformulation comprises separating viable and non-viable cells prior to a subsequent refreezing. Density gradient centrifugation is preferably employed for this purpose. For example, a 30% PERCOLL® gradient centrifugation procedure may be employed (Madan, A. et al. (1999) "Effect of Cryopreservation on Cytochrome P-450 Enzyme Induction in Cultured Rat Hepatocytes, Drug Metab. Dispos. 27(3):327-335; Sun, E. L. et al. (1990) "*Cryopreservation Of Cynomologus Monkey* (*Macaca fascicularis*) *Hepatocytes For Subsequent Culture And Protein Synthesis Studies,*" In Vitro Cell Development and Biology 25:147-150; Lawrence, J. N. et al. (1991) "*Development Of An Optimal Method For The Cryopreservation Of Hepatocytes And Their Subsequent Monolayer Culture,*" Toxicology In Vitro, 5(1):39-51; Dou, M. et al. (1992) "*Thawed Human Hepatocytes In Primary Culture,*" Cryobiology 29(4):454-469; Utesch, D. et al. (1992) "*Characterization Of Cryopreserved Rat Liver Parenchymal Cells By Metabolism Of Diagnostic Substrates And Activities Of Related Enzymes,*" Biochemical Pharmacology 44:309-315. For example, the thawed cells may be resuspended in a prewarmed (approximately 37° C.) 30% PERCOLL® isotonic fractionation buffer and then centrifuged at 100×g at room temperature for twenty minutes to pellet viable cells. The supernatant is discarded and the cells are resuspended in media for a subsequent cryopreservation step directly or for further processing prior to cryopreservation.

Cryopreserved preparations that result from the freezing of a previously frozen-thawed preparation will preferably have a post-thaw cell viability of greater than 50% and more preferably 70% or more. Such high viabilities enable the present invention to accomplish the repeated freezing and thawing of hepatocytes without unacceptable losses of cells or the need for ever greater samples sources.

Pooled Hepatocyte Preparations

The capacity of the present invention to enable the repeated freezing and thawing of hepatocytes additionally facilitates the production of pooled hepatocyte preparations, especially pooled human hepatocyte preparations. As discussed above, individual liver samples yield hepatocytes having differing metabolic capabilities. In order to facilitate the reproducible use or study of hepatocytes, it is desirable to minimize hepatocyte differences attributable to such sample variation by pooling hepatocytes from different sources to obtain a composite or "average" hepatocyte preparation. Such composite hepatocyte preparations may thus be formulated so as to provide a preparation having the metabolic activities of an "average" hepatocyte sample or a preparation whose hepatocyte enzyme functions approximate the hepatocyte enzyme functions of freshly isolated hepatocytes. Such metabolic activities may include, for example, some or all of the following enzymatic activities: coumarin 7-hydroxylase (COUM), dextromethorphan O-demethylase (DEX), 7-ethoxycoumarin O-deethylase (ECOD), activities responsible for the phase II metabolism of 7-hydroxycoumarin (7-HCG and 7-HCS), mephenytoin 4-hydroxylase (MEPH), testosterone 6(β)-hydroxylase (TEST), tolbutamide 4-hydroxylase (TOLB), phenacetin O-deethylase (PHEN), or chlorzoxazone 6-hydroxylase (CZX). The substrates, methods of measurements and assay units for assays of such metabolic activities are provided in Table I.

For example, preferred preparations of pooled hepatocytes will yield assay values within the ranges identified in Table II. Alternatively, the hepatocytes samples used to form the pooled preparation may be selected so as to maximize, minimize, or emphasize certain hepatocyte functions over other functions so as to yield a pooled preparation that exhibits a user desired profile of liver cell function(s).

The pooled hepatocyte preparations of the present invention may comprise hepatocytes obtained from the same source at differing times, or from two or more different sources. Preferably, pooled hepatocyte preparations will result from the pooling of hepatocytes obtained from three, four, five, six or more different sources.

Most preferably, the pooled hepatocyte preparations of the invention will comprise at least one population of hepatocytes that were cryopreserved prior to pooling. For example, a pooled hepatocyte preparation may comprise one or more hepatocyte specimens that were cryopreserved prior to pooling with one or more freshly isolated hepatocyte specimens. Alternatively, a pooled hepatocyte preparation may comprise only hepatocyte specimens that were previously cryopreserved. Table II provides the normal range (i.e., the range between Assay Minimum and Assay Maximum for each Assay). Table II values are derived data of the last 150+ lots of human cryopreserved hepatocytes

TABLE I

Hepatocyte Metabolic Activities

Abbreviation	Substrate/Assay	Method of Measurement	Units
7-HCG	7-hydroxycoumarin glucuronide	Phase II metabolism of 7-hydroxycoumarin	pmol/min/10^6 cells
7-HCS	7-hydroxycoumarin sulfate	Phase II metabolism of 7-hydroxycoumarin	pmol/min/10^6 cells
NAT1	p-aminobenzoic acid	N-acetylation of p-aminobenzoic acid	nmol/mg/min
NAT2	Sulfamethazine	N-acetylation of Sulfamethazine	nmol/mg/min
VBTY	viability	Trypan Blue ™ exclusion	percentage
AP	Alkaline Phosphatase	Sigma kit	units/mg protein
GGT	gamma-glutamyl transpeptidase	GGT stain	positive
UGT1	7-hydroxycoumarin	Phase II metabolism of 7-hydroxycoumarin	169 pmol/mg/min
P450	cytochrome p450 content	carbon monoxide difference spectrum	Not determined for cryo hepatocytes nmol/mg protein
CZX	chlorzoxazone	chlorzoxazone 6-hydroxylation	31.1 pmol/mg/min*
COUM	coumarin	coumarin 7-hydroxylation	50.0 pmol/mg/min*
DEX	dextromethorphan	dextromethorphan O-demethylation	21.4 pmol/mg/min*
MEPH	mephenytoin	mephenytoin 4-hydroxylation	24.1 pmol/mg/min*
PHEN	phenacetin	phenacetin O-deethylation	28.9 pmol/mg/min*
TEST	testosterone	testosterone 6(beta)-hydroxylation	96.8 pmol/mg/min*
TOLB	tolbutamide	tolbutamide 4-hydroxylation	30.6 pmol/mg/min*
PROT	protein content	Pierce protein kit	Not determined for cryo hepatocytes mg/mL
ECOD	ethoxycoumarin	7-ethoxycoumarin O-deethylation	37.3 pmol/mg/min*

TABLE II

Hepatocyte Assays

Assay	Normal Range (pmol/min/10^6 cells)
coumarin 7-hydroxylation	1 to 154
Dextromethorphan O-demethylation	0.5 to 96
7-ethoxycoumarin O-deethylation	1 to 154
Phase I metabolism of 7-hydroxycoumarin	2 to 545
Phase II metabolism of 7-hydroxycoumarin	0 to 110
mephenytoin 4-hydroxylation	0.2 to 442
testosterone 6(beta)-hydroxylation	2 to 675
tolbutamide 4-hydroxylation	1.8 to 82
phenacetin O-deethylation	1 to 125
chlorzoxazone 6-hydroxylation	2 to 215

In certain embodiments of the invention, hepatocyte preparations will have assay values in the above stated ranges for at least three of, and preferably for at least four of, still more preferably for at least six of, and most preferably for at least eight of the following assays: the COUM assay; the DEX assay; the ECOD assay; the 7-HCG assay; the 7-HCS assay; the MEPH assay; the TEST assay; the TOLB assay; the PHEN assay; the CZX assay.

If desired, the cryopreserved hepatocytes may be plated on to collagen-coated tissue culture plates, or tissue culture plates coated with other extracelluar matrix proteins including but not limited to laminin, fibronectin, entactin, poly-L-lysine, gelatin, or any combination thereof. Preferably, this is accomplished by diluting an appropriate volume (e.g., 0.2 ml to 2.5 ml) of diluted cells (e.g., cells having a concentration of approximately 0.7×10^6 cells/ml) onto the plates. For plating on a 96-well microtiter plate, it is desirable to further dilute the cell suspension to a concentration of 0.35×10^6 cells/ml with InVitroGRO CP medium, and to add 100 μl of the cell suspension to each well. It is preferred to even distribute the cells in the wells. This can be accomplished by gently shaking the plates in a back-and-forth and side-to-side manner; the use of a circular motion will cause the cells to unevenly pool in the center of the wells. Human hepatocytes handled in this manner will attach to the plates in 2-4 hours, however, if minimal handling is desired, the cells can be allowed to attach overnight.

Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention unless specified.

EXAMPLE 1

Refreezing of Thawed Hepatocyte Preparations

Cryopreserved hepatocytes are thawed and refrozen as indicated below.

Materials: 30 cc syringe, two couplers, PERCOLL® in a 1 L bag, INVITROGRO™ CP-2 medium in a 2 L bag, 1-2 L autoclaved beaker, heater, waterbath, centrifuge tube rack.

Procedure:

1) Set up a recirculating waterbath heater at 37-42° C.

2) Add approximately 200-400 mls of INVITROGRO™ CP medium to a 1-2 L beaker. Equip Biological Safety Cabinet with a manual pipet or a liquid handling robot.

3) Remove approximately 50 cryovials from dewar receptacle and quickly place them in 2 test tube racks. When possible, space vials apart.

4) Submerge solid cell suspensions into heated waterbath until the ice chunks can be dislodged when the vial is inverted.

5) Pour cell suspension from each vial into the beaker. Add 1 ml of INVITROGRO™ CP medium from the small beaker into each vial to rinse and pour contents into the beaker. Transfer thawed cell suspension into a 1 L sterile bag.

6) Attach bag, INVITROGRO™ CP medium in a 2 L bag and PERCOLL® bagged at 30% onto COBE® automated cell processor(a centrifugal system incorporating a flexible membrane that allows the removal of fluids while spinning through use of a rotating seal, hydraulic pump and flexible membrane) and process according to standard practices.

7) Perform a cell count.

8) Cryopreserve cell suspension.

PERCOLL/REDIGRAD™ (Amersham Biosciences) is employed for the PERCOLL® density centrifugation. PERCOLL® is composed of colloidal silica coated with polyvinylpyrrolidone (PVP). The REDIGRAD™ formulation is also composed of colloidal silica but is covalently coated with silane. These coatings are thought to render the material non-toxic and ideal for use with biological materials. Both particles have a density of 1.13 g/ml. Centrifugation of sample in the presence of PERCOLL/REDIGRAD™ results in the spontaneous formation of a density gradient due to the heterogeneity of particle sizes in the medium.

PERCOLL/REDIGRAD™ is best used in balanced salt solutions, such as physiological saline (0.15 M NaCl), although 0.25 M sucrose may be employed. The addition of 9 parts (v/v) of PERCOLL/REDIGRAD™ to 1 part (v/v) of either 1.5 M NaCl, 10× concentrated cell culture medium, or 2.5 M sucrose will result in a solution adjusted to about 340 mOs/kg H_2O. Solutions of different osmotic pressure can be produced by adjusting the relative volumes of PERCOLL/REDIGRAD™ and salt or sucrose solution. (Vincent, R. et al. (1984) "*Adjustment Of The Osmolality Of Percoll For The Isopycnic Separation Of Cells And Cell Organelles,*" Anal. Biochem. 141(2):322-328). The final adjustment to the required osmolality can be carried out by the addition of salts or distilled water. Concentrations other than 10× physiological saline may also be used satisfactorily.

PERCOLL/REDIGRAD™ will form self-generated gradients by centrifugation in fixed-angle rotor heads after 15 minutes. Hepatocytes can be separated by centrifugation at 50-100 g_{av} in fixed-angle or swinging bucket rotor heads after 10-30 minutes.

EXAMPLE 2

Variation of Primary Hepatocyte Samples

To illustrate the sample-to-sample variation of different sources of individual (unpooled) hepatocytes, hepatocytes are isolated from 82 different donors and analyzed for cell viability and enzyme function. The following metabolic activities are evaluated: COUM, DEX, ECOD, 7-HCG, 7-HCS, MEPH, TEST, TOLB, PHEN, and CZX. The results are shown in Table III.

TABLE III

Variation of Hepatocyte Samples

Lot No.	Sex	% V	COUM	DEX	ECOD	7-HCG	7-HCS	MEPH	TEST	TOLB	PHEN	CZX
067	M	62%	67	1	70	231	24	2	44	35	27	24
086	F	74%	51	23	10	50	9	1	38	13	BQL	18
089	F	77%	25	21	8	23	6	1	11	13	BQL	29
090	F	74%	30	25	7	13	BQL	2	19	15	BQL	16
091	F	73%	13	29	66	44	10	12	252	36	27	36
094	F	67%	41	12	37	24	4	21	126	40	19	87
099	F	86%	21	15	7	4	BQL	1	60	10	BQL	22
104	M	81%	63	21	44	247	25	2	58	37	8	20
105	M	67%	59	15	24	38	14	1	29	27	12	27
110	F	77%	45	24	35	23	4	6	206	11	54	36
111	F	71%	4	10	9	2	3	3	147	2	19	19
114	F	75%	39	23	21	10	5	5	59	TBD	3	45
122	M	79%	26	30	29	80	5	1	42	23	4	25
129	F	90%	4	24	27	67	18	1	16	33	10	51
ACU	F	81%	53	8	25	74	18	8	80	16	4	23
AIT	F	83%	45	29	13	118	14	BQL	82	15	11	7
AOK	M	73%	60	21	58	283	64	5	86	30	24	21
ATR	M	73%	7	11	1	39	11	BQL	11	8	3	4
AVF	M	70%	59	13	50	210	29	BQL	54	37	11	24
BTP	M	88%	66	29	36	214	25	3	50	45	11	12
CEC	M	86%	47	26	17	105	27	21	32	57	38	36
CEK	F	80%	55	2	39	141	5	16	302	41	7	16
CHD	F	77%	30	14	53	471	42	4	28	23	13	26
CPN	M	81%	28	6	40	100	13	2	168	14	37	21
ECM	M	85%	8	11	10	55	18	6	81	18	21	9
EFA	M	69%	9	9	35	47	5	18	66	12	47	67
EHI	F	90%	88	3	56	291	45	1	248	21	45	43
EJR	F	75%	89	14	32	288	43	8	62	41	1	41
ENR	M	73%	69	28	27	124	31	107	77	36	38	32
EOB	M	88%	14	11	18	65	9	31	49	14	21	35
ETR	F	88%	30	1	34	13	13	7	13	13	37	69
EVY	M	80%	2	20	23	218	77	33	24	25	17	38
FNL	M	85%	17	27	62	282	32	6	6	50	14	24
FRW	M	75%	46	22	16	106	17	48	25	54	44	8
GBE	F	77%	5	49	31	165	20	2	16	19	5	54
GNG	F	74%	57	17	33	54	8	5	95	22	16	22
GTV	F	71%	32	6	8	47	7	BQL	40	28	16	11
GUY	M	92%	65	12	11	73	12	20	90	13	5	8
HHG	M	83%	2	8	14	251	29	BQL	28	12	4	40
HRU	M	90%	43	28	39	175	15	4	69	40	57	44
ICJ	M	74%	134	20	60	287	17	BQL	129	82	28	7
IEM	M	88%	34	17	23	129	34	72	48	23	19	34
IHR	F	76%	17	43	8	84	9	9	95	46	41	7
LID	M	86%	36	31	50	307	54	1	142	49	21	51
IRX	F	73%	57	5	40	172	24	12	113	43	6	18
JUL	M	82%	7	11	3	41	9	7	23	12	2	3
KK5	M	83%	1	8	27	319	38	BQL	61	17	17	42
KPT	F	83%	9	12	32	248	30	55	65	26	56	32
KRJ	F	76%	6	40	76	359	37	1	11	61	23	20
KRM	F	78%	126	36	55	83	17	103	98	46	74	44
K5E	M	73%	65	27	52	206	74	21	123	42	93	16
KZO	F	82%	38	16	38	262	8	6	75	32	30	20
LAE	M	76%	58	15	50	294	22	14	67	63	125	21
MOF	F	91%	79	17	29	10	12	2	85	5	7	46
MRS	M	72%	119	21	110	450	50	2	675	54	68	28
MTR	F	69%	2	33	23	218	3	5	38	67	39	8
MYO	F	94%	40	24	9	24	BQL	BQL	12	7	BQL	11
NPX	F	79%	36	32	13	130	6	15	76	25	20	10
NQT	M	85%	76	12	39	80	23	2	151	14	20	34
OAU	F	81%	47	26	24	86	8	6	85	46	53	13
OZL	M	76%	16	15	61	300	109	3	165	29	17	43
PFM	F	87%	21	1	11	67	10	3	116	8	15	33
PXK	M	80%	86	35	63	433	78	2	109	62	32	60
QWG	F	77%	16	32	29	300	21	9	50	10	BQL	15
REL	F	77%	40	20	15	109	9	65	100	33	75	10
RFA	F	78%	130	42	49	444	52	6	195	30	28	17
RKB	F	95%	42	16	16	100	8	3	36	17	20	19
RML	F	76%	BQL	6	45	129	31	14	152	24	42	29
RNG	F	91%	119	14	97	298	27	177	207	34	71	41
ROE	F	82%	73	24	36	302	37	2	55	17	2	51
SEO	F	72%	36	25	18	106	9	66	102	50	81	11
SQJ	F	74%	115	12	100	285	19	175	210	30	81	42
SRA	M	79%	50	6	71	409	84	10	23	28	18	44
TPZ	F	83%	120	13	101	301	26	171	204	31	82	41

TABLE III-continued

Variation of Hepatocyte Samples

Lot No.	Sex	% V	COUM	DEX	ECOD	7-HCG	7-HCS	MEPH	TEST	TOLB	PHEN	CZX
TSR	F	62%	47	66	58	175	20	BQL	6	77	16	34
VCM	M	82%	42	28	79	415	110	0.2	94	16	4	215
VEN	F	70%	79	69	89	328	53	2	32	58	48	81
VTA	M	78%	32	1	25	84	12	5	120	21	40	15
WWM	M	84%	42	1	27	127	12	6	58	21	16	37
ZAG	M	85%	35	28	39	96	73	1	11	42	17	18
ZCR	M	80%	84	11	39	160	22	38	14	57	20	18
ZIJ	M	72%	6	33	31	320	29	13	25	34	3	13

BQL (Below Quantitation Limit)
TBD (To Be Determined)

EXAMPLE 3

Characterization of Pooled Hepatocytes

Cryopreserved pooled lots of hepatocytes are prepared and analyzed for post-thaw viability and enzyme function. The following metabolic activities are evaluated: COUM, DEX, ECOD, 7-HCG, 7-HCS, MEPH, TEST, TOLB, PHEN, and CZX.

Six lots of pooled hepatocytes, comprising either five-donor pools or ten-donor pools, are prepared as described above. Hepatocytes are harvested from individual donors and then cryopreserved as individual lots using liquid nitrogen as freezing agent. Cryopreservation is accomplished by suspending the hepatocytes into freezer-safe vials containing a medium having approximately 10% DMSO and approximately 90% Cryopreservation Medium. The dispensed hepatocytes are then frozen in a controlled rate freezer until a final temperature of approximately −80° C. is reached.

To form the pooled hepatocyte preparations, individual lots are thawed, and the viable cells are isolated by percoll gradient centrifugation. Vials of individual donor cryopreserved hepatocytes were thawed in a 37° C. waterbath (perhaps it would be better to give a range such as 30-40° C. waterbath?) for 60-90 seconds. The thawed cells are decanted into 37° C. media containing 30% Isotonic Percoll and 70% CP-2 media. The cell suspension is centrifuged at 100 g for 20 minutes. The viable cells are recovered in cryopreservation media and counted. The viable cells are diluted to 20 million cells per mL. A second solution containing 20% DMSO and 80% cryopreservation media (equal volume to the cell suspension listed above) is prepared. The 20% DMSO and 80% cryopreservation media is slowly added to the cells suspension mixture. The addition takes 5-10 minutes. The resulting mixture is 10% DMSO, 90% cryopreservation media with cells at 10 million cells per mL. This solution is aliquoted into cryovials at 1.0 mL per vial. The cells are then cryopreserved. Viable cells from individual lots are then pooled to form pooled hepatocyte preparations whose cells have functional assay values within desired ranges.

The pooled lots are then cryopreserved. Table IV below shows the results of the post-thaw viability ("% V") and enzyme function analysis of the pooled lots. As indicated in Table III, pools had an average viability of 79% (S.D. ±6%).

TABLE IV

Summary of Pooled Hepatocyte Lot Data

Pool	% V	COUM	DEX	ECOD	7-HCG	7-HCS	MEPH	TEST	TOLB	PHEN	CZX
MJI[a]	89	63	28	66	301	44	12	70	61	50	62
YDJ[a]	72	66	30	80	470	41	1	165	31	27	71
APO[b]	79	79	21	55	276	43	2	112	22	26	30
HMB[b]	76	61	18	70	231	46	3	151	23	20	83
IJU[b]	75	32	19	35	232	44	2	124	32	11	42
RKS[b]	81	84	20	73	336	53	2	131	28	23	55

[a]5-donor lots
[b]10-donor lots

For comparison, Table V below shows summary data for a post-thaw viability and enzyme function analysis of eighty-one individual lots that are cryopreserved (i.e., subjected to one cycle of cryopreservation). This data confirms that the lot-to-lot variability of enzyme function found in individual hepatocyte sources is very high. The data confirms the desirability of employing pooled hepatocyte preparations for providing cryopreserved cells that approximate the enzyme function of "average" hepatocytes for a wide variety of enzymes.

TABLE V

Summary of Pooled Hepatocyte Lot Data

	% V	COUM	DEX	ECOD	7-HCG	7-HCS	MEPH	TEST	TOLB	PHEN	CZX
Avg	79	37	17	51	276	27	8	35	35	15	19
High	95	134	69	110	471	110	177	675	82	125	215
Low	62	6	1	31	231	24	2	25	34	3	13

EXAMPLE 3

Characterization of the Viability of Pooled Hepatocytes After Thawing

A common use of for cryopreserved hepatocytes is to thaw the hepatocytes and then incubate them with various xenobiotics. For this purpose, it is preferred that the hepatocytes maintain their viability for at least several hours. To examine the post-thaw viability over time for one lot of pooled cryopreserved hepatocytes, the cells were thawed, aliquoted into the wells of a 12-well plate, and incubated at 37° C. with 5% CO_2. The viability of the hepatocytes is then measured at time-points for up to six hours. Table VI shows the results of this analysis, wherein, at six hours, 39% of the hepatocytes remained viable

TABLE VI

Post-Thaw Viability Analysis of a Pooled Hepatocyte Lot

Timepoint	% Viability [a]
T = 0	88%
0.5 hrs	79%
1.0 hrs	84%
2.0 hrs	79%
3.0 hrs	73%
4.0 hrs	67%
6.0 hrs	69%

[a] viability determined by Trypan Blue

All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth.

What is claimed is:

1. A method of producing a desired preparation of multi-cryopreserved hepatocytes, said hepatocytes, being capable of being frozen and thawed at least two times, and in which greater than 70% of the hepatocytes of said preparation are viable after the final thaw, said method comprising:

(A) subjecting hepatocytes that have been frozen and thawed to density gradient fractionation to separate viable hepatocytes from non-viable hepatocytes,

(B) recovering the separated viable hepatocytes, and

(C) cryopreserving the recovered viable hepatocytes to thereby form said desired preparation of hepatocytes without requiring a density gradient step after thawing the hepatocytes for the second time, wherein the hepatocytes are not plated between the first and second cryopreservations, and wherein greater than 70% of the hepatocytes of said preparation are viable after the final thaw.

2. The method of claim **1**, wherein said density gradient fractionation comprises density centrifugation through polyvinylpyrrolidone-coated colloidal silica particles.

3. The method of claim **1**, wherein said hepatocytes are selected from the group consisting of human hepatocytes, porcine hepatocytes, simian hepatocytes, canine hepatocytes, feline hepatocytes, bovine hepatocytes, equine hepatocytes, ovine hepatocytes and rodent hepatocytes.

4. The method of claim **3**, wherein said hepatocytes are human hepatocytes.

5. The method of claim **1**, wherein said preparation comprises a pooled preparation of hepatocytes of multiple sources.

6. The method of claim **5**, wherein said multiple sources are of the same gender, race, or health state.

7. The method of claim **5**, wherein the hepatocytes of said pooled preparation of hepatocytes provide said pooled preparation with a desired level of a metabolic activity.

8. The method of claim **7**, wherein said metabolic activity is selected from the group consisting coumarin 7-hydroxylase (COUM), dextromethorphan O-demethylase (DEX), 7-ethoxycourmarin O-deethylase (ECOD), activities responsible for the phase II metabolism of 7-hydroxycoumarin (7-HCG and 7-HCS), mephenytoin 4-hydroxylase (MEPH), testosterone 6(β)-hydroxylase (TEST), tolbutamide 4-hydroxylase (TOLB), phenacetin O-deethylase (PHEN), and chloroxazone 6-hydroxylase (CZX).

9. The method of claim **1**, wherein greater than 80% of the hepatocytes of said preparation are viable.

10. A method of investigating in vitro drug metabolism comprising incubating hepatocytes of a multi-cryopreserved hepatocyte preparation in the presence of a xenobiotic, and determining the metabolic fate of the xenobiotic, or the affect of the xenobiotic on the hepatocytes or on an enzyme or metabolic activity thereof, wherein the hepatocytes have been frozen and thawed at least two times, and wherein greater than 70% of the hepatocytes of said preparation are viable without requiring a density gradient step after thawing the hepatocytes for the second time, wherein the hepatocytes are not plated between the first and second cryopreservations.

11. The method of claim **5**, wherein said multiple sources are of different gender, race or health state.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,604,929 B2
APPLICATION NO. : 11/110879
DATED : October 20, 2009
INVENTOR(S) : Daniel Dryden et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page, item (*) Notice: should read as follows: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 433 days.

At column 19, line 26, please change "39%" to --69%--.

Signed and Sealed this

Fifteenth Day of June, 2010

David J. Kappos

David J. Kappos
Director of the United States Patent and Trademark Office

US007604929C1

(12) EX PARTE REEXAMINATION CERTIFICATE (8874th)

United States Patent

Dryden et al.

(10) Number: US 7,604,929 C1

(45) Certificate Issued: *Feb. 28, 2012

(54) **CELLULAR COMPOSITIONS AND METHODS FOR THEIR PREPARATION**

(75) Inventors: **Daniel Dryden**, Westminter, MD (US); **James Hardy**, Ijamsville, MD (US)

(73) Assignee: **KBC Bank NV**, London (GB)

Reexamination Request:
No. 90/011,276, Oct. 8, 2010

Reexamination Certificate for:

Patent No.:	**7,604,929**
Issued:	**Oct. 20, 2009**
Appl. No.:	**11/110,879**
Filed:	**Apr. 21, 2005**

(*) Notice: This patent is subject to a terminal disclaimer.

Certificate of Correction issued Jun. 15, 2010.

(51) **Int. Cl.**

A01N 1/00	(2006.01)
C12N 5/00	(2006.01)
C12N 5/08	(2006.01)

(52) **U.S. Cl.** **435/1.1**; 435/1.3; 435/370; 435/374; 435/375

(58) **Field of Classification Search** ..... None
See application file for complete search history.

(56) **References Cited**

To view the complete listing of prior art documents cited during the proceeding for Reexamination Control Number 90/011,276, please refer to the USPTO's public Patent Application Information Retrieval (PAIR) system under the Display References tab.

Primary Examiner—Sharon Turner

(57) **ABSTRACT**

The present invention relates to novel cell (e.g., hepatocyte, etc.) compositions and methods for their preparation and use. In particular, the invention concerns methods of processing preparations of such cells so as to permit their repeated cryopreservation and thawing while retaining substantial viability. The invention also concerns preparations of cells (e.g., hepatocytes) that have been repeatedly cryopreserved and thawed.

EX PARTE REEXAMINATION CERTIFICATE ISSUED UNDER 35 U.S.C. 307

THE PATENT IS HEREBY AMENDED AS INDICATED BELOW.

AS A RESULT OF REEXAMINATION, IT HAS BEEN DETERMINED THAT:

The patentability of claims **1-9** and **11** is confirmed.

Claim **10** is cancelled.

* * * * *